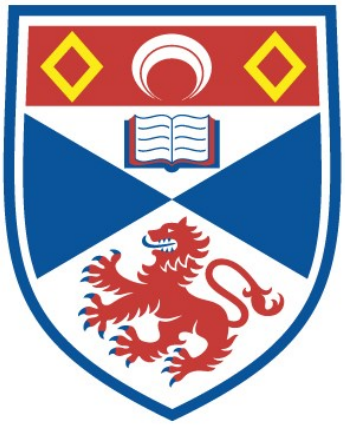
# ETHANOL PRODUCTION BY ZYMOMONAS MOBILIS CP4 UNDER CHEMOSTATIC AND CONTINUOUS TRANSIENT REACTOR OPERATION

João Batista Buzato

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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# ETHANOL PRODUCTION BY *ZYMOMONAS MOBILIS* CP4 UNDER CHEMOSTATIC AND CONTINUOUS TRANSIENT REACTOR OPERATION

A thesis presented by

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in application for the degree of

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## ABSTRACT

Ethanol production by *Zymomonas mobilis* CP4 under chemostatic and continuous transient reactor.

Ethanol production by *Zymomonas mobilis* CP4 under chemostat and continuous transient operation has been investigated.

Under carbon limitation with simple chemostatic operation, glucose, fructose and sucrose were tested at 35° C. At 2%(w/v) carbon source medium, glucose was more efficiently utilised. Ethanol production values for glucose, fructose and sucrose were respectively: volumetric productivity 2.5, 1.8 and 2.5 g/l/h; conversion to ethanol efficiency 96, 88 and 76% at values of dilution rate of 0.35, 0.21 and 0.32 $h^{-1}$ which represent approximately 85% of $D_m$. With 5%(w/v) glucose values of volumetric productivity of 5 g/l/h and conversion to ethanol efficiency of 85% were achieved. However, under nitrogen limitation with glucose 5%(w/v), approximately 25% of feed glucose medium was being washed out while conversion to ethanol efficiency was around 60% at a dilution rate of 0.17 $h^{-1}$

Under alternating glucose concentrations of 2 and 5%(w/v) medium and at a fixed dilution rate of 0.2 $h^{-1}$, continuous transient operation achieved values of ethanol conversion higher than 80%. However, both continuous transient (utilising high alternating glucose concentration of 8 and 11% and 8 and 16%(w/v)) and simple chemostatic operation running with 9.5 and 12%(w/v) glucose medium were less satisfactory as conversion efficiency values were as

less satisfactory as conversion efficiency values were as low as 40 and 29% for the former and 48 and 45% for the latter.

Pulsing the culture with mineral salts ($NH_4^+$, $Mg^{++}$, $K^+$) and ethanol, the culture showed no effect with mineral salts and a strong inhibitory effect on growth for ethanol.

## CERTIFICATE

I hereby certify that João Batista Buzato has spent nine terms engaged in research work under my direction and that he has fulfilled the conditions of Ordinance General Nº 167 of the Resolution of the University Court 1967, Nº 1, and that he is qualified to submit the Accompanying thesis for the degree of Doctor of Philosophy.

## DECLARATION

I hereby declare that this thesis is based on work carried out by me, that the thesis is of my own composition and that no part of it has been presented previously for a higher degree.

The research was conducted in the Department of Biochemistry, University of St. Andrews, under the direction of Dr. W. M. Ledingham.

## ACADEMIC RECORD

I graduated with the degree of Pharmacy and Biochemistry in 1980 from the University of Maringá, Paraná, Brazil and with the degree of Master of Biological Science in 1985 from the University Julio de Mesquita Filho - Campus Rio Claro, São Paulo, Brazil.

I matriculated as a research student in the Department of Biochemistry, University of St. Andrews, in October 1988.

## ACKNOWLEDGMENT

I should like to express my gratitude to Dr. W. M. Ledingham for his advice during this work, to Brazilian folks and Mr. McManus for their incentive and friendship.

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CONTENTS

# 1. INTRODUCTION

## 1.1 SCIENCE AND TECHNOLOGY IN BRAZIL

Although Pedro Alvares Cabral, a Portuguese sea captain in 1500 drove upon Brazil coast and declared it as a possession of the Portuguese crown, it was not until the 19th century that one can talk about science and technology in that country. Because of the Napoleon war and consequently the invasion of Portugal, the Prince Regent D. Joao decided to take refugee in Brazil with the royal family. There were artists, intellectuals and scientists accompanying them. They arrived in 1808 and in that year the Prince Regent abolished by decree all the limitations which had been put on Brazilian industry. The printing press was established and institutions of science and technology were created, such as The Medical School in Rio de Janeiro and Bahia, The National Library and The National Museum. D. Joao also elevated Brazil from the category of colony to The United Kingdom of Brazil and Portugal. At that time, Brazil was seen as an exporter of natural products, such as minerals and food. (5).

At the beginning of the 20th century, a number of scattered faculties and institutions of research were developed between Rio de Janeiro and Sao Paulo, due to their economic and political power.(43) Over that period waves of immigrants from Italy, Germany, Japan, Poland and Portugal settled mainly in areas of coffee plantations of The Southeast and South to develop the

region (1). Industrial production rose particularly in Sao Paulo but all technology and the majority of qualified staff and scientists were foreign. There had been no interest to develop a core of Brazilian scientists. (11).

It was only in 1912 when the first Brazilian university was created in Parana, followed by Rio de Janeiro in 1920 and Minas Gerais in 1927. As a matter of the fact, these institutions were a group of several isolated professional schools. Nevertheless, science and technology did improve in Brazil when the University of Sao Paulo (USP) was created in 1934 (5).

The foreign technology adoption is a feature in different phases of the Brazilian industrialization process (62). Until World War II, technology produced abroad arrived in the country in the form of goods and machinery with qualified people to work on it.

A new phase started in the 50s, when the industrialization process of Brazil accelerated. This phase characterized by expansion and diversification due to import substitution and in the large use of foreign technology in production areas. Meanwhile, some institutions were created to provide finantial support for research. The two first research council - The National Council for Scientific and Technological Development (CNPq) and The Council to improve the training of higher education teaching staff (CAPES) were created in 1951 (5).

In 1956, Brazilian president Juscelino Kubitschek launched his administration programme aiming at a faster development which favoured private investment and opened the country to foreign penetration by transnational corporations, through offers of tax incentives and easy profit remission. However, due to the massive state spending on the construction of Brasilia and the road system, inflantion had risen by 1961 to nearly 50% per year. (43) As result, a sucession crisis followed, with the emergence of new forms of popular and worker organization. In response to that, in 1964 the military forces took power and installed their regime. It was only 21 years later that a civil elected president took office again.

The boom known as the "Brazilian miracle" started in 1968. The government subsidised the purchase of farm machinery and the agricultural sector became a leading consumer of industrial products and exporter earner. However, it was again prefered to import technology rather than to develop it locally.

### 1.1.1 PROALCOOL - THE BRAZILIAN ALCOHOL PROGRAMME

The sharp rises in the price of crude oil in the early 70s had a deep effect in the economy of Brazil as well as investment in science and technology.

The import-oil based Brazil economy was seriously threated. The country imported 85% of its need of oil in 1973 (65), which shows its great dependence on foreign supply.

Fuel expenditure raised from a tenth of the country's total imports in 1970, to more than a half, a decade later.

In a effort to stem the haemorrhage of foreign exchange the government decided in 1975 to launch a project to replace gasoline with locally-produced ethanol-alcohol distilled from sugar cane.

Before World War II several governments subsidised the production of industrial ethanol from crop such as corn, potatoes and sugar beet. These subsidies were intended to reduce dependence on oil imports in the event of war and to encourage home industry and agriculture and reduce trade deficits during the depression.

During World War II, most motor vehicles in Germany ran on potato-based ethanol. From 1945 onwards the decline in subsidies, substantial increases in the price of molasses due to its use in cattle feed and the availability of cheap ethanol based on petrochemical ethylene led to a rapid decline in production by fermentation.

Brazil has a rapidly rising population and another intention of the programme was to provide jobs to reduce unemployment and support the sugar industry at a time of world sugar surplus.

Ethanol production industry currently employs some 300,000 people in farming, and 90,000 in sugar factories and distilleries,and has helped to minimize the drift of

population into towns. The number of jobs indirectly denpendent on ethanol production may be nearly one million. (67)

The use of ethanol as fuel has proved to be far less polluting than gasoline. Cars burning alcohol produce 57% less carbon monoxide, 74% fewer hydrocarbons and 13% less nitrogens oxides than engines fired by fossil fuels (79).

In 1975, Brazilian production of ethanol from sugar cane was only 903 million l/yr, mainly for industrial purposes. But, benefiting from government subsidies, the programme picked up speed, and all gasoline used in the country was rapidly converted to "Brazilian gasohol", a mixture of up to 20% ethanol and 80% gasoline.

In 1979, the Iran-Iraq war threatened Middle Eastern oil supplies, and car industry, stimulated by the government, took the bold step of producing cars with new engines adapted for the use of hydrated ethanol.

In 1981, Brazilian production of ethanol reached a total of 4.08 billion liters, of which 1.88 billion were consumed as hydrated ethanol in more than 300,000 out of 8 million automobiles. The remainder of the fleet used 2.2 billion liters of anhydrous ethanol mixed with gasoline in the proportion 10 - 20% ethanol (79).

The Proalcool Programme has influenced sponsorship in science and technology. In agriculture scientists have achieved important gains in productivity. The Technology

Research Centre in Piracicaba (Sao Paulo) has succeeded in boosting sugar-cane production by 18% by breeding a superior cane plant(6).

One problem faced by researchers was stillage, the highly-polluting waste liquid which was regularly flushed into rivers killing both fish and other kinds of aquatic life. The Brazilian ethanol programme generates waste equivalent to one and a half times the sewage produced by the entire Brazilian population. Eventually, the waste turned out to be both an excellent fertilizer for sugar cane, when well-defined amounts of it are used in the soil (98), and an effective biological pesticide.

Eventhough oil is a depletable resource and its overuse will lead to exhaustion, nowadays the future of the programme is uncertain. Brazilian oil production has increased and the country imports about half of the oil it needs.(65). Furthermore the oil price has decreased drastically and the programme has no longer viable prospects because alcohol can only compete with gasoline when the international oil price is US$ 40 a barrel. Nevertheless, ethanol production has reached 12 billion l/yr (1).

However, the low technology approach adopted by alcohol distilleries was introduced in the decade of 1940 and remains unchanged, in spite of the incredible technological developments in petrochemical fractionation methods (21). As result, alcohol distillery industrial still have various aspects which can be improved in

order to be an efficient alternative industry producing a competitive fuel.

Although struggling with the highest foreign debt the world has ever known, Brazil government has invested money in the universities and other research centres as a strategy of development.

In this context, opportunities have been provided for scientists and technologists to acquire specialized skills with the aim of developing a suitable technology to the Brazilian reality.

## 1.2 THE BACTERIA *ZYMOMONAS MOBILIS*

In general, most carbohydrate feedstock has been fermented with the use of yeasts. However, some attention has been given to the possibilities of using organisms other than yeasts to produce ethanol. The advantages sought are increased conversion yields to ethanol, faster fermentation rates, higher operating temperatures to reduce expenditure on cooling and the ability to grow on a wider range of substrates.

The bacteria *Zymomonas mobilis* seems to be an efficient ethanol producer and many papers have discussed both physiological and industrial aspects.

### 1.2.1 CARBOHYDRATE METABOLISM IN *ZYMOMONAS MOBILIS*

Carbohydrate metabolism in *Zymomonas mobilis* has been extensively studied due to the considerable potential for industrial alcohol fermentations. This

bacteria can utilize only three carbohydrates: glucose, fructose and sucrose.

The ability of *Zymomonas* to metabolize glucose and fructose was first reported by Baker and Hillier (8) and then by Lindner (56).

A molar equation for the conversion of glucose to ethanol was established by Kluyver and Hoppenbrouwers (62 ) as follows:

1 glucose $\longrightarrow$ 1.8 ethanol + 1.9 $CO_2$ + 0.15 lactic acid.

Fructose conversion to ethanol is comparable to glucose (81,101). Ethanol production was also observed when this sugar comes from enzymatically hydrolysed inulin (the polymer found in *Jerusalem artichoke*).

The formation of by-products such as acetaldehyde, acetate, lactate, acetoin, and glycerol have been reported. These end products are principally formed under aerobic conditions. (60,59)

More recently, Viikari and Korhola (107) have proposed routes of by-products formation in *Zymomonas mobilis*, for glucose and fructose metabolism.

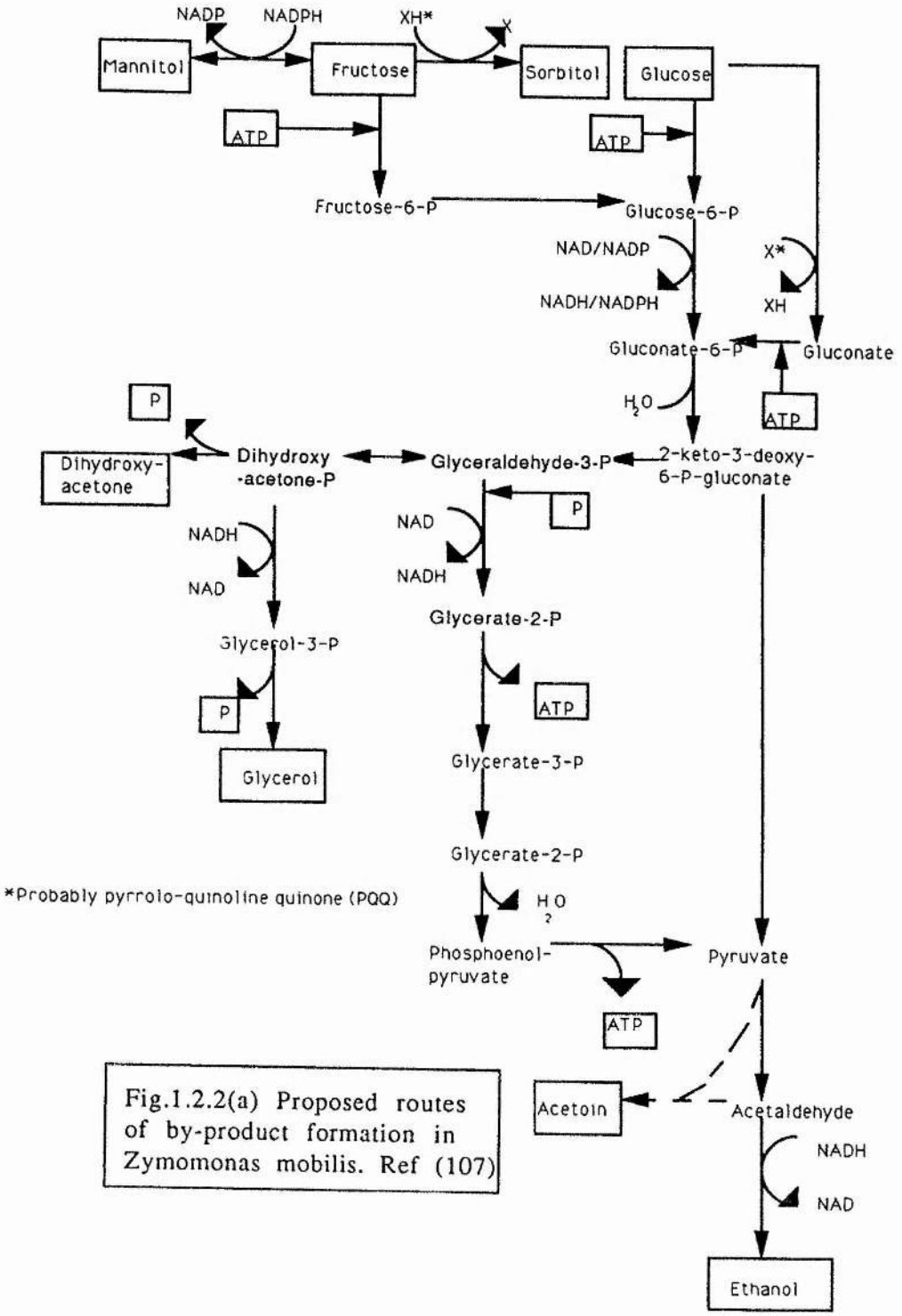



Fig.1.2.2(a) Proposed routes of by-product formation in Zymomonas mobilis. Ref (107)

Important research has been carried out to elucidate the absorption of sugar preceding its catabolism.

Romano et al. (84) have demonstrated that *Zymomonas* lacks a PEP-glucose phosphotransferasae system and a permease system but the presence of a glucose facilitated diffusion system for glucose has been reported (28). Earlier evidence of Belaich et al. (15) indicated that glucose transport was twofold greater than glucose utilized. Therefore the rate of glucose catabolism was not limited by glucose permeation and they suggested that a carrier mediated or facilitated diffusion transport system was the only method of sugar uptake.

More recently Lyness and Doelle (57,58) have reported that during growth on sucrose, fructose is always taken up by the cell more slowly than glucose. A further study of glucokinase and fructokinase by Doelle (30,29) showed that glucokinase is inhibited by ADP, AMP, glucose-6-phosphate, and high concentrations of nucleotide triphosphate (ATP, UTP, and CTP). In other words, this enzyme is regulated by the energy status of the cell. However, fructokinase reacts only with fructose and ATP and is inhibited by glucose and glucose-6-phosphate. Thus the presence of glucose regulates the uptake of fructose. This author (30) has proposed that these two enzymes for sugar transport were independently controlled. Glucokinase is controlled by ATP and ADP (e.g., cellular energetics), and fructokinase, which is not affected by the nucleotides , is severely repressed by

glucose. Any disturbance in the energy status of the cell will affect glucokinase first and the accumulation of glucose will automatically control fructokinase activity.

The first step of sucrose breakdown should almost unavoidably produce glucose or fructose or both. However, experiments of Ribbons et al. (80) and McGill et al. (60) showed that the molar growth-yield coefficients for sucrose were appreciably lower than those for the equivalent concentrations for glucose plus fructose. It was also observed that centrifugation of sucrose cultures yielded a cell pellet and an opalescent supernatant fluid. This cloudy substance could be precipitated by ethanol 75%.Further analysis led to the discovery of levan. So fructan formation could explain the diversion of some of the energy source.

The results of Dawes et al. (26) showed that approximately 10% of the sucrose utilized by crude extracts of *Zymomonas mobilis* was converted into levan, the remainder of the sucrose was hydrolyzed to glucose and fructose. They did not estabilish whether or not an invertase was present and suggested tentatively that a levansucrase could accounted for both levan formation and sucrose hydrolysis.

More recently, in addition to the high molecular weight levan, which is precipitable by ethanol 75%, low molecular levans or fructo-oligomers have also been reported. Viikari and Gisler (106) have reported that the amount of low molecular weight fructo-oligomers was found even to exceed that of precipitable levan (high molecular weight fructo polymer).

It was in the early 1950's, Gibbs and DeMoss (35) discovered that *Zymomonas mobilis* utilized the Entner-Doudoroff pathway anaerobically for glucose and fructose metabolism in association with a pyruvate decarboxylase. The fate of the majority of the individual C atom is:

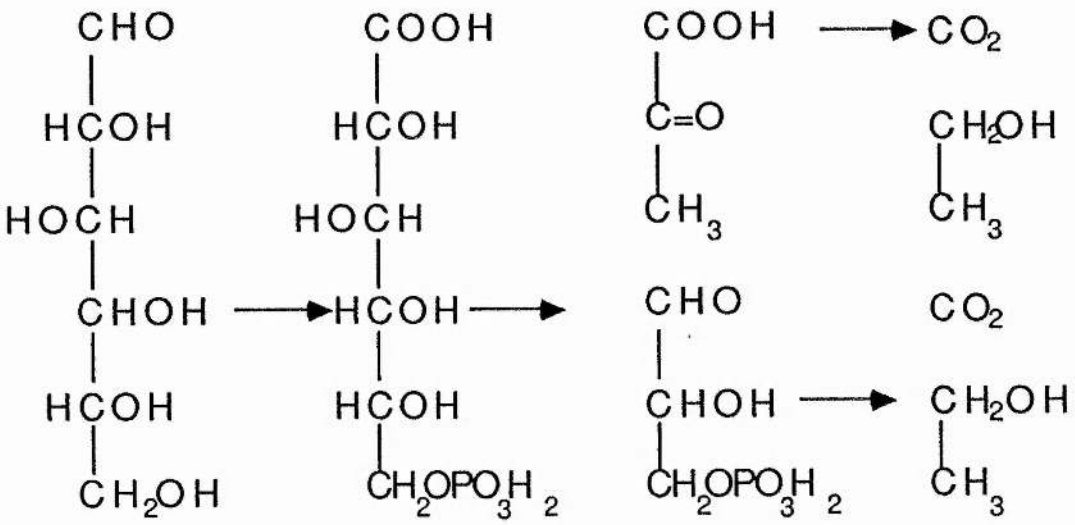



Figure 1.2.2(b) The path of individual atoms during the metabolism of hexose by Zymomonas mobilis Ref.(35)

The presence of the Entner-Doudoroff pathway in *Zymomonas mobilis* has been confirmed at the enzymatic level by several authors.

Doelle (30) has published the existence of two separate constitutive enzymes for glucose and fructose.

Raps and DeMoss (78) have demonstrated that cell-free extracts fermented glucose-6phosphate to ethanol and $CO_2$. The extracts contained phosphohexoisomerase, and a dehydrogenase for glucose-6-phosphate.

According to Cromie and Doelle (23), as *Zymomonas mobilis* solely utilises the Entner-Doudoroff pathway of glucose, whereby the sugar is split into pyruvate and glyceraldehyde-3-phosphate, half of the molecule always leads to ethanol. However, the fate of glyceraldehyde-3-phosphate depends on the demand for 4- and 5-carbon precursors for RNA and DNA biosynthesis or ATP energy and ethanol production. Montenecourt (66) has emphasized the idea that a large portion of glyceraldehyde-3-phosphate must be converted to pyruvate considering the near theoretical value of ethanol production.

There are two enzymes involved in the conversion of pyruvate to ethanol, pyruvate decarboxylase, and alcohol dehydrogenase.

Pyruvate decarboxylase has been found in crude extracts by MacGill et al. (60) and MacGill and Dawes (59).

Hoppner and Doelle (41) have studied alcohol dehydrogenase and suggested that the characteristics of this enzyme are very similar to those of the yeast enzyme.

A detail scheme of the Entner-Doudoroff pathway utilized by *Zymomonas mobilis* is given.in figure 1.2.2.(c)

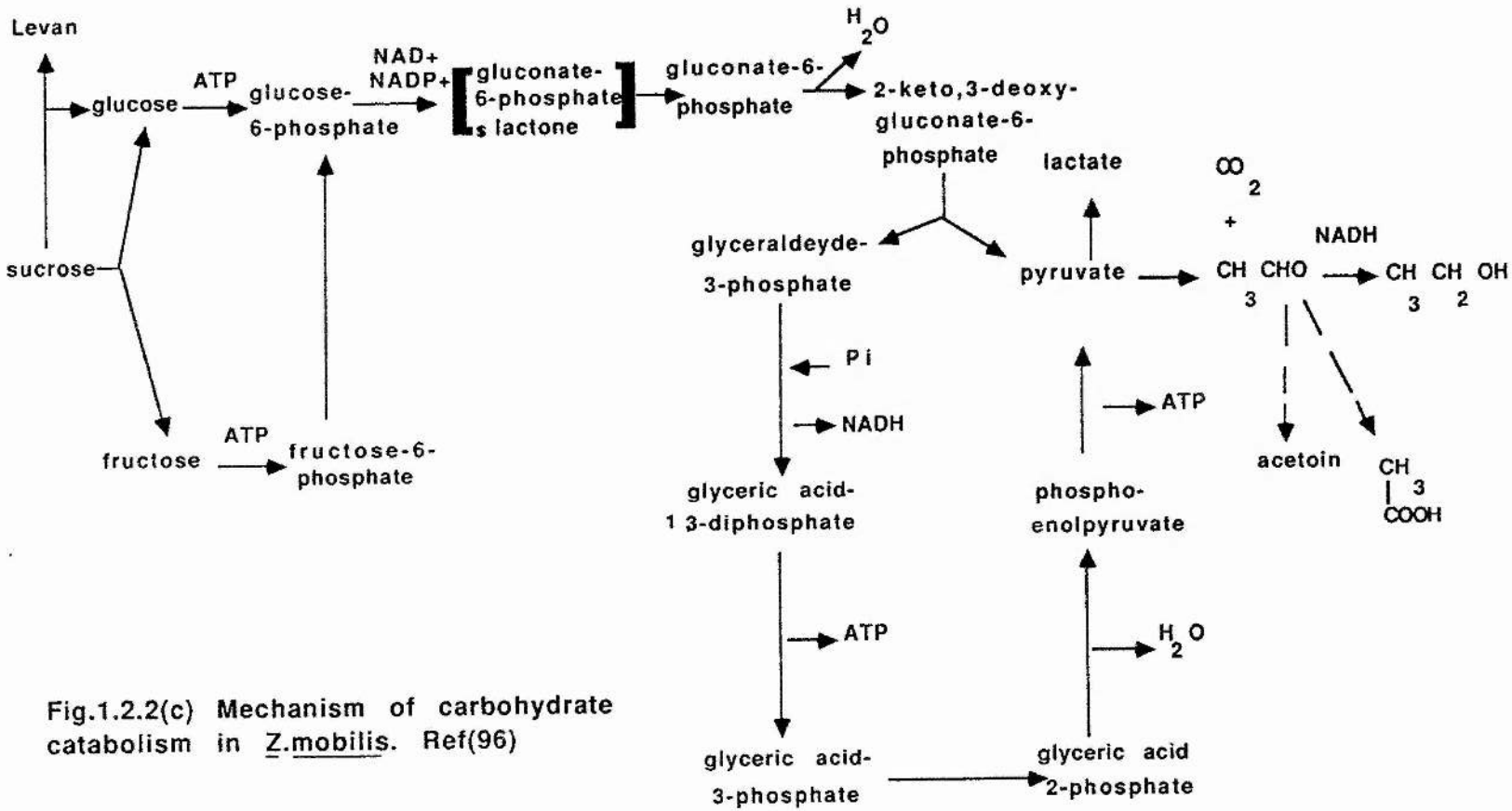



Fig.1.2.2(c) Mechanism of carbohydrate catabolism in Z.mobilis. Ref(96)

## 1.2.2- CONTINUOUS CULTURE

The continuous culture of microrganisms is a technique of well-known importance in both microbiology studies and more recently as large scale production process. The essential feature of this technique is that microbial growth occurs under steady-state, which means, growth happens at a constant rate in a constant environment. Factors such as pH values, concentrations of nutrients, metabolic produts, which inevitably change when a batch culture is used, are all maintained constant or independently controlled by the experimenter (40).

A continuous fermentation (chemostat) consist of a mixed suspension of cells into which fresh medium is continuously added at a constant rate and the culture is harvested at the same rate so that the culture volume is kept constant.

The biomass growth is limited by the concentration of a single limiting substrate, while all the other nutrients are in excess.

A schematic representation of a chemostat is shown in the figure 1.2.3 (a).

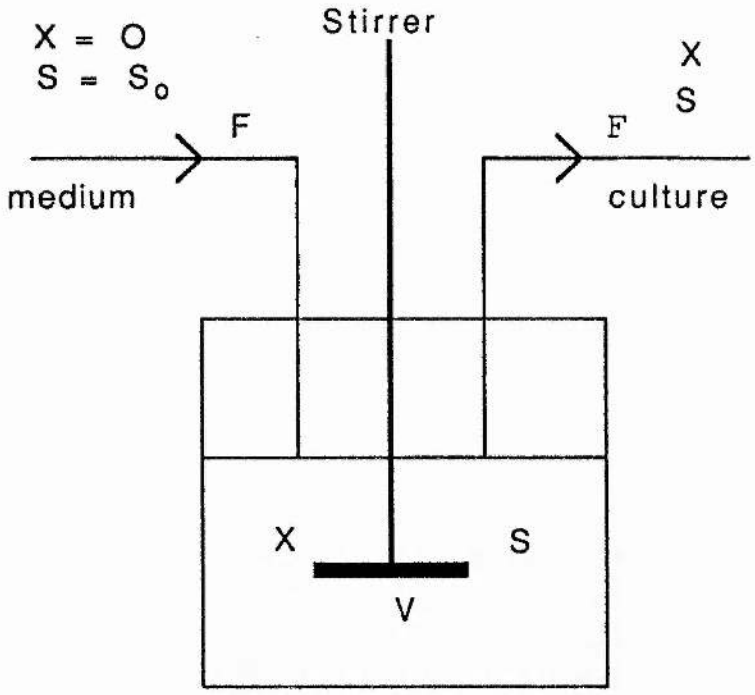



Fig.1.2.3(a) The chemostat (diagramatic). The biomass and growth-limiting substrate concentrations are represented by X and S respectively. $S_o$ is the limiting substrate in the feed medium; F= Flow Rate; V= Culture Volume Ref.(75)

The flow of medium into the vessel is related to its volume by the term dilution rate (D) as:

$$D = F/V$$

That is the number of culture volumes of medium passing through the growth vessel per unit time, the dimension being reciprocal time ($h^{-1}$).

**Wash-out rate.** Assume for the moment that the bacteria in the culture vessel are not growing or dividing . With complete mixing, every organism in the vessel has an equal probability of leaving within a given time. The wash-out rate, i.e. the rate at which organisms initially present in the vessel would be washed out if growth ceased but flow continued is given by:

$$-dx/dt = Dx \qquad (1).$$

where x is the concentration of organisms in the vessel.

**Bacterial growth kinetics.** Suppose that, at first, growth is allowed to proceed batchwise without addition of medium. Following a period during which the growth rate of the cells gradually increases, the cells grow at a constant, maximum rate and this is as the log, or exponential phase. The exponential phase may be described by the equation:

$$dx/dt = \mu x \qquad (2).$$

Where x is the concentration of organisms at time t, $\mu$ is the specific growth rate. In this equation $\mu$ is usually assumed to be constant. However this assumption is correct

only when all substrates necessary for growth are present in excess.

Monod (88) first showed that there is a simple relationship between the specific growth rate and the concentration of an essential growth substrate, $\mu$ being proportional to the substrate concentration when this is low but reaching a limiting saturation value at high substrate concentrations according to the equation:

$$\mu = \mu_{max}\ S/(K_s+S) \qquad (3).$$

where S is the substrate concentration, $\mu_{max}$ is the growth rate constant (i.e. the maximum value of $\mu$ at saturation levels of substrate) and $K_s$ is a saturation constant numerically equal to the substrate concentration at which $\mu = 1/2\ \mu_{max}$.

Monod (88) also showed experimentally that there was a constant relationship between the growth of a culture and substrate utilization.

$$dx/dt = -Y(ds/dt) \qquad (4).$$

where Y is termed the yield factor. Over any finite period of time during the exponential growth phase, the yield (Y) is equal to weight of bacteria/weight of substrate consumed.

**Changes in concentration of organisms.** In the chemostat the organisms are growing at a rate described by equation (2) and simultaneously being washed away at a rate determined by the equation (1).

The net change in concentration of biomass with time will be determined by the relation :

$$\text{increase} = \text{growth} - \text{outflow}$$

$$dx/dt = \mu x - Dx.$$

$$dx/dt = x(\mu - D) \quad (5).$$

It follows that if $\mu > D$, dx/dt will be positive and the concentration of organisms in the culture will increase with time. If, however, $\mu < D$, then dx/dt will have a negative value, and the cell concentration will diminish with time: the culture is washed out from the growth vessel. Only when $\mu = D$ will $dx/dt=0$ and the concentration of organisms in the culture remains constant with the time. This condition is called steady state. Under such steady state conditions, the specific growth rate, $\mu$, of the organisms in the culture vessel is exactly equal to the dilution rate D. Provided the dilution rate is maintained constant, the system is self-balancing.

This relationship between the growth rate of the organism and the dilution rate makes it possible to adjust the growth rate of the organism, within certain limits, to any value desired. When the dilution rate is increased above the specific growth rate, $D > \mu$, then dx/dt becomes negative, the biomass decreases, and the substrate is not utilized and increases. With $\mu < \mu_{max}$, the increased substrate concentration positively influences the specific growth rate up to its limit, where the specific growth rate equals the maximum growth rate ($\mu = \mu_{max}$). However, the specific growth

rate, can not be made to exceed $\mu_{max}$ and therefore steady state conditions can not be obtained at dilution rates above a critical value ($D_c$), which is nearly equal to $\mu_{max}$. If the dilution rate is set to a value greater than $D_c$, the bacteria will be washed out of the culture vessel faster than they can grow. Therefore the critical value of the dilution rate is of great practical importance.

**Changes in substrate concentration.** The substrate enters the growth vessel at concentration $S_0$, is consumed by the organisms, and emerges in the overflow at a concentration S. Therefore, the net change in substrate concentration is :

increase of substrate = input - output - consumption

$$ds/dt = DS_0 - DS - (\text{growth/Yield})$$

↑ from equation (4)

$$ds/dt = D(S_0 - S) - \mu x/Y \quad (6).$$

Rearranging the equation (6) and substituting $\mu$ by equation (3)

$$ds/dt = D(S_0 - S) - \{\mu_{max} x/Y\}\{S/(K_s + S)\} \quad (7).$$

Similarly $\mu$ can be substituted for equation (5)

$$dx/dt = x\{[\mu_{max} S/(K_s + S)] - D\} \quad (8).$$

The equations (7) and (8) define the behaviour of the culture in the chemostat. They also show that the continuous culture, in spite of the initial state of the culture, should finally establish a steady state.

Because equations (7) and (8) equal zero in a steady state, the values of x and S, which will be designated x and S can be calculated for the conditions of the steady state. For the calculation of x, equation (7) is used

$$D(S_0 - S) = \{\mu_{max} x/Y\}\{S/(K_s + S)\}$$

Since in the steady state $D = \mu_{max}\{S/(K_s + S\}$

$$\bar{x} = Y(S_0 - \bar{S}) \qquad (9)$$

The concentration of the $\bar{S}$ is then determined from equation (8)

$$\mu_{max} S/(K_s + S) = D$$

$$\bar{S} = DK_s/(\mu_{max} - D) \qquad (10)$$

Substituting $\bar{S}$ from equation (10) in equation (9), it follows that

$$\bar{x} = Y\{S_0 - K_s[D/(\mu_{max} - D)]\}$$

As a production process, the total output from a continuous culture unit, is equal to the product of dilution rate and concentration of microorganisms. There is a value of D for which the product $D\bar{x}$ is a maximum; in other words, for any system there is a particular dilution rate, $D_m$, which gives the maximum output of microorganisms without excessive substrate being left in the effluent.

The figure 1.2.3(b) shows the relationship between bacterial concentration ($\bar{x}$), substrate concentration ($\bar{S}$), doubling time (dt), and bacterial output ($D\bar{x}$), at different dilution rates (D) in a chemostat.

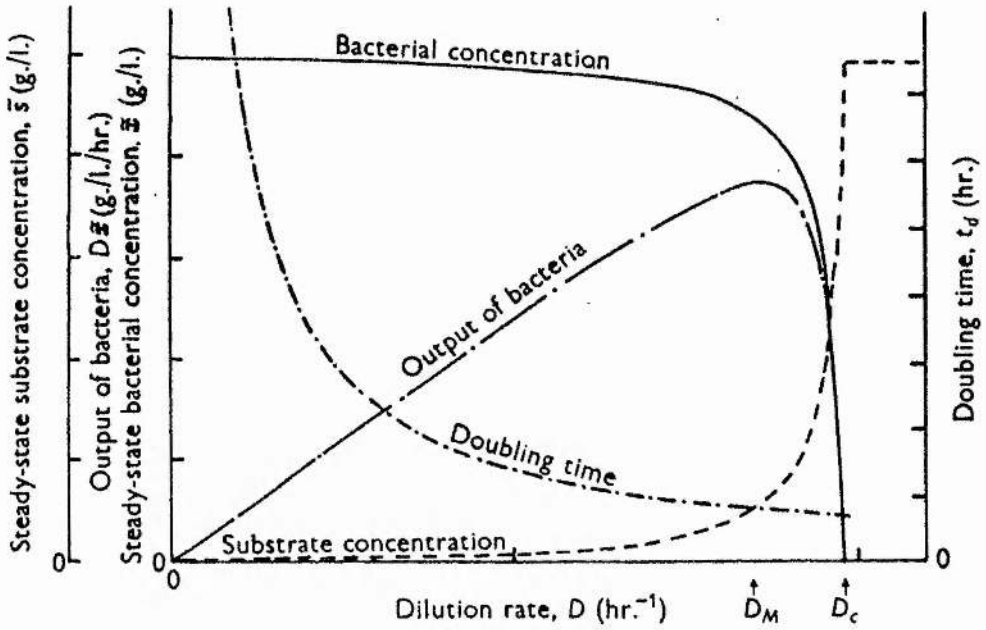



Figure 1.2.3(b) *Relationships between bacterial concentration, substrate concentration, doubling time, and bacterial yield in the steady state, at different dilution rates (D) in a chemostat.* Ref (40)

### 1.2.2.1 *ZYMOMONAS MOBILIS* AND CONTINUOUS FERMENTATION.

Numerous works have been devoted to continuous fermentation for the production of ethanol via on yeast (55,94,54,61). However,recently a significant portion of research in this area has been based on *Zymomonas mobilis.* Some of the advantages of the *Zymomonas* process reported in studies are:

(a) Significantly higher specific rates of sugar uptake and ethanol production compared with those found for yeasts (46);

(b) Considerably higher volumetric ethanol productivities (48);

(c) Higher ethanol yields and lower biomass production than for yeasts (83);

(d) *Zymomonas* cultures grow anaerobically , but unlike yeasts do not require a small (but controlled) supply of oxygen to maintain both viability and hence ethanol productivity at high cell concentrations (57).

(e) The genetic manipulation of *Zymomonas* should be simpler than for yeasts (88).

### 1.2.2.2 CONTINUOUS FERMENTATION OF GLUCOSE BY *ZYMOMONAS MOBILIS*.

Lee et al. (53) have reported studies utilising *Zymomonas mobilis* in continuous culture with 10, 15 and 20% glucose media. At 10% glucose, steady state conditions were achieved under glucose-limited conditions. However when

higher concentrations were used, the sugar was not fully metabolized even at low dilution rates and oscillatory behaviour was shown. It was proposed that ethanol inhibition of growth was responsible for this phenomena. The strain used ATCC 10988 also showed a relatively high proportion of glucose used for maintenance energy probably due to a high concentration of glucose in the culture vessel, increased osmotic pressure and increased ethanol concentration in a 15% a system.

Lee et al. (50) have also reported studies with *Zymomonas mobilis* ZM4 with 6, 10, 13.5 and 17% glucose media. It was evident that for those media with lower concentrations of glucose, the cultures behaved as a glucose-limited chemostat at low dilution rates. However, when 17% glucose medium was used, the glucose was in excess at all dilution rates tested. This was taken to indicate that the growth was ethanol-limited. This strain showed to be superior to strains ATCC 10988 as both the specific growth rate and the specific ethanol production rate were higher and this strain was less influenced by the concentration of ethanol in the medium.

Lee et al. (52) have claimed that the ethanol inhibition effect is enhanced at high temperature (37° C) for the strain ZM4 at dilution rate 0.10 $h^{-1}$.

The fermentation of glucose based on *Zymomonas mobilis* in continuous culture has also been reported by Lavers et al.(45). These researchers have used the strain ATCC 29191 and 10% glucose medium. According to them, due to inhibitory

effect of the alcohol, the fermenter could not be operated under conditions of glucose-limitation at values of dilution rate higher than 0.20 $h^{-1}$.

Cromie and Doelle (23) have established optimal growth conditions for strain Z10, a laboratory strain developed from ATCC 10988, using continuous cultivation methods that concentrations of various mineral components were used and supplemented with yeast extract or pantothenic acid. Although it certainly appears that magnesium plays a major role in the passage of glucose across the cell membrane, increasing its amount did not improve glucose utilization. It was found that growth factor requirements were satisfied with the addition of yeast extract but were not with the addition of pantothenate alone.

The renewed interest in ethanol as a liquid fuel has stimulated research and development into continuous fermentation process. The publication of Bringer and Sahm (17) is a case in point. These researchers have reported fermentation conducted on a 50 $m^3$ scale utilising industrial waste starch fraction (B starch) which is formed in the course of the production of glucose syrup from wheat flour. This starch was first enzymatically hydrolyzed and produced 12% (w/v) of glucose and the process run at dilution rate of 0.07 $h^{-1}$. The main problem was the appearance of acid lactic and a decrease of fermentation productivity after 2-3 days of continuous operation. However, in several runs steady-state conditions were re-established after 24-30 hours and the fermentations proceeded over time periods of up to 20 days.

Baratti et al. (9) have isolated a flocculent strain of *Zymomonas mobilis*. The isolation was done in a conical fermenter at high dilution rate of 0.5 $h^{-1}$. This new isolate named ZM4F JM1 was able to ferment 10% glucose medium at dilution rates from 0.1 to 2 $h^{-1}$. The ethanol concentration was higher than 40 g/l up to a dilution rate of 1.5 $h^{-1}$. The geometry of the fermentor allowed the biomass concentration to reach high values (15-20 g/l).

The knowledge of factors influencing the morphology and performance of *Zymomonas* are of importance in the development of commercially successful fermentations and of novel types of bioreactors.

Stevnsborg and Lawford (91) have studied the effect of calcium in chemostat culture of *Zymomonas* in 10% glucose plus salts medium. Phase microscopy of the samples revealed filamentous growth at calcium concentrations higher than 30 mM. The effect of calcium to induce filament formation was noticed to be enhanced by $CO_2$. The authors have suggested that although calcium influences the growth morphology, it does not have any effect on either the growth rate or the biomass yield..

Stevnsborg et al. (93) have reported that increasing the temperature in chemostat culture of *Zymomonas* with low and high glucose concentrations caused a decreasing frequency of septations leading to the formation of long filaments and regions of the outer membrane that were thought to be disrupting. This observation has suggested that the arrest of septation and cell division was due to temperature-

sensitive enzymes involved in septation. When 2% glucose medium was used, the elevating of the temperature from 30° C to 35° C caused a five fold increase in the maintenance energy coefficient but did not appreciably affect the maximum growth yield.It was also observed that operating the fermenter at elevated temperature of 33° C instead of 30° C in chemostat with 10% glucose appeared to be advantageous since there was an increase in both the rate of glucose uptake and the rate of ethanol formation. However further increases in temperature resulted in a high effluent glucose concentration at a dilution rate of 0.24 $h^{-1}$.

Stevnsborg and Lawford (92) have published a comparative study of strain ATCC 31821 and its mutant ATCC 31823 using a medium containing 10% (w/v) glucose. The mutant strain ATCC 31823 was select from a nitrosoguanidine-mutagenized culture of ATCC 31821 by virtue of its growth and survival in 15% (v/v) ethanol. The conclusion of these researchers was that no significant differences were seen between the two strains as both strains showed a similar performance at a fixed dilution rate Of 0.10 $h^{-1}$.

More recently, the parental strain (ATCC 31823), which was originally isolated in Zaire, has been studied by Schmidt and Schügerl (85) to produce ethanol in continuous culture in the presence of aspartate as co-substrate to avoid nitrogen limitation. The glucose concentrations used were 6, 10 and 15% (w/v). The results showed that cell mass and ethanol concentrations diminish with high values of dilution rates and substrate inhibition started at 70-80 g/l glucose. No product inhibition was found at ethanol

concetrations of up to 70 g/l. Furthermore the cell mass yield coefficient showed to be dependent on dilution rate and on maintenance coefficient according to a non-linear relationship unlike cultivations with complex media for which a linear relationship exists between yield coefficient and dilution rate . It was also observed that the maintenance coefficient becames larger with increasing substrate and/or product concentration.

1.2.2.3 CONTINUOUS FERMENTATION OF FRUCTOSE BY *ZYMOMONAS MOBILIS*

By far the greater proportion of published work using *Zymomonas mobilis* for ethanol production has used glucose as substrate. This substrate can be obtained from polymers such as starch and cellulose after a hydrolysis process. However another source of fermentable sugar which can be utilized by these bacteria is fructose. Fructose can be obtained from renewable agricultural sources, tubers of *Jerusalem artichoke* are a case in point.

Toran-Diaz et al. (100) have studied the influence of pH and medium composition when fructose 10% (w/v) is the substrate using batch culture. When yeast extract at 0.5%(w/v) in the medium composition was used, very little growth occured at pH 4.0, but for pH 5 to 6.5 the maximum biomass were about the same (1.7 g/l) and values of ethanol were also kept the same (42 g/l). When yeast extract was replaced by panthothenate, at 5 x $10^{-3}$ g/l, cell yield was as high as with yeast extract; while the specific growth

rate was lower. The final ethanol concentration kept the same.

Toran-Diaz et al. (102) have used strain ZM 4 in studies carried out in continuous culture. The results showed that maximum values of biomass and ethanol were respectively 0.7 g/l and 46 g/l at dilution rate below 0.07 $h^{-1}$. However the percentage of fructose conversion dropped sharply from 97% to 48% when the dilution rate was increased to 0.09 $h^{-1}$.

Allais et al. (3) have described the conversion of enzymatically hydrolyzed of inulin from tubers of *Jerusalem artichoke* to ethanol using strain ZMF4, a flocculent mutant from strain ATCC 31821. The sugar concentration was adjusted at 100 g/l and a fermenter with a internal settler in order to achieve higher concentrations of biomass was used. As a result, biomass reached 22 g/l which is much higher than the concentration observed with a non flocculent strain. Residual sugar concentration was low (less than 1.6 g/l) for dilution rates lower than 1 $h^{-1}$ and then increased slowly to 16 g/l. The ethanol concentration decreased slowly from 50 g/l to 35 g/l at high dilution rate (2.5 $h^{-1}$).

1.2.2.4 CONTINUOUS FERMENTATION OF SUCROSE BY *ZYMOMONAS MOBILIS*.

The principal substrate for ethanol fermentation in use in Brazil is sucrose from sugar cane. It is a renewable and relatively inexpensive source of sucrose and sugar cane is an old and traditional crop, linked with economic history of Brazil and widely cultiveted through out the country on a large scale.

Sugar cane offers a potential yield of over 4,000 litres of ethanol per hectare per annum (67), the highest for any crop, and the energy balance for ethanol production is more favourable than with other crops due to the contribution obtainable by burning the bagasse, the remains after extracting the sugar.

Because of the low price of sugar on the world market the opportunity cost using it for ethanol production is low. Furthermore Brazil has the necessary conditions for a substantial increase in sugar cane production due to its large areas suitable for expansion of the crop and technical support by experimental stations with the capacity and experience to develop increasingly productive hybrids, high in sugar content and disease resistence (74).

However ethanol production based on *Zymomonas mobilis* and sucrose leads to reduction of ethanol yield. This is caused by the formation of two majors by-products. Levan was reported as early as 1962 (80) while the production of sorbitol was reported more recently (104).

In batch culture, when an equimolar mixture of glucose and fructose is used for ethanol production, sorbitol is formed as a major by-product and it accounts for 11% of the original substrate (105). When ethanol is produced from sucrose, the formation of levan and sorbitol reduces the ethanol yield to 80% of theoretical values while it is at least 94% of theoretical if glucose or fructose is used (100,81).

Since sucrose hydrolysis can be easily performed using a soluble or immobilized invertase to treat a natural source of sucrose such as cane juice or molasses, higher ethanol yields are expected on hydrolyzed sucrose because levan formation can not occur and sorbitol formation is expected to be low.

Favela Torres and Baratti (31) have reported the effect of dilution rate and sugar concentration on the kinetic and yield parameters of ethanol production from an equimolar mixture of glucose and fructose by a flocculent strain of *Zymomonas mobilis* using a fermenter with a internal settler. Their results showed that, at a concentration of 100 g/l, an equimolar mixture of glucose and fructose was efficiently converted into ethanol. At dilution rates up to 0.8 $h^{-1}$ the ethanol yield (0.5 g/g) was higher than that observed on sucrose (0.43 g/g). This high ethanol yield was mainly the result of low sorbitol concentration observed and the absence of levan formation. At dilution rate higher than 0.8 $h^{-1}$ the ethanol yield decreased as a result of sorbitol formation. In parallel, the fructose uptake rate decreased while glucose uptake was unaffected. As a consequence fructose accumulated faster than glucose in the fermenter. With the use of a flocculent strain and a fermenter with a internal settler, the biomass reached 12 g/l. Figures of biomass and ethanol (10.5 and 48 g/l) were constant respectively for sugar concentrations from 100 to 140 g/l at a constant dilution rate of 0.4 $h^{-1}$. Both concentrations decreased in presence of 160 g/l of equimolar mixture of glucose and fructose. Sorbitol production increased linearly

with sugar concentrations and reached a maximal value of 12 g/l. Fructose accumulated in the broth faster than glucose and the ethanol yield decreased from 0.49 to 0.30 $g.g^{-1}$.

Lee et al. (51) have grown *Zymomonas mobilis* ZM4 on 10% and 15% sucrose (w/v) in continuous culture. In both sucrose concentrations, there was full utilization of sugar and no observable levan formation at dilution rate values lower than 0.1 $h^{-1}$. However higher dilution rate ( 0.15 - 0.30 $h^{-1}$ ) led to significant and increasing concentration of sucrose and fructose together with levan formation. Their results showed that for 15% sucrose (w/v) medium, excess of sucrose was present at all dilution rates and the system functioned as a product-limited continuous culture at a concentration of up 60 g/l ethanol. A reduction in biomass yield was observed (compared to growth on glucose) which was attributed to levan formation.

Lawford et al. (46) have used 10% sucrose (w/v) medium and *Zymomonas mobilis* ATCC 29191. At dilution rates less than 0.2 $h^{-1}$ a yield of 0.4g ethanol/g sucrose was reached with complete utilization of glucose (the residual sugar being sucrose and fructose) and very little levan formation. These authors have also attempted to use molasses and the sucess has been limited to medium containing yeast extract (1.5% w/v)and 10% fermentable sugar. The maximum dilution rate value, for complete sugar to ethanol conversion, was only 0.1 $h^{-1}$.

Viikari and Linko (108) have given a very important contribution for the understanding of sucrose fermentation

by *Zymomonas mobilis*. According to their results the conversion rate of sucrose was, in no case, the limiting step in its utilization. Even at the highest dilution rate (0.3 $h^{-1}$) or highest sucrose concentration (20% w/v), only up to 4% of the original sucrose remained in the medium. However theoretical ethanol yields were only obtained when sucrose was at 12% (w/v) and at low dilution rates. When the dilution rate exceeded 0.15 $h^{-1}$. glucose and fructose began to accumulate in the medium. Levan formation increased sharply when the dilution rate exceeded 0.1 $h^{-1}$, but remained almost constant at dilution rates higher than 0.18 $h^{-1}$. When sucrose was used at 20% (w/v) and the dilution rate at 0.18 $h^{-1}$, there was an accumulation of glucose and fructose and the ethanol yield was only around 50% of the theoretical.

Other parameters of sucrose fermentation such as pH, temperature and substrate concentration have been studied by Favela Torres and Baratti (31). In their publication a flocculant strain of *Zymomonas mobilis* and a cell recycle fermenter with a settler were used. With 10% sucrose medium, biomass concentrations in the range 8 - 13 g/l were obtained in the fermenter that was operated at dilution rates from 0.2 to 1.2 $h^{-1}$. Sucrose was extensively hydrolysed up to dilution rate of 1.0 $h^{-1}$ but dropped to 91% at 1.2 $h^{-1}$. Glucose and fructose were present in the fermenter at increasing concentrations with dilution rate and ethanol values were 40g/l which represents 79% of theoretical but decreased sharply at 1.2 $h^{-1}$ value of dilution rate. The reduced ethanol yield was attributed to formation of

byproducts like levan. In their publication a dilution rate of 0.4 $h^{-1}$ was selected to study the effect of sucrose concentration. The biomass concentration and levan formation were slightly increased from 12 to 13.6 g/l and 10 to 13 g/l, respectively while the ethanol reached 60 g/l for a sucrose concentration of 15% in the medium. The fermentation was carbon-limited up to 12.5% sucrose medium.

In regarding to temperature their main conclusions were that most of the kinetic and yield parameters were not affected in the range of 30 - 36° C at fixed dilution rate of 0.4 $h^{-1}$. Sucrose hydrolysis was not rate limiting nor was glucose or fructose utilization. This might have been the result of the tolerance to temperature of the strain used and the use of cell recycle. Since the levan formation was not lowered increasing the temperature therefore the ethanol was not increased either.

In their last set of tests, their results showed that at temperature of 30° C sucrose fermentation could be operated from pH 4,0 to 5,5 with only slight changes in the kinetics and yield parameters. However at pH 6,0 the sucrose hydrolysis was rate limiting.

### 1.2.2.5- CONTINUOUS ETHANOL PRODUCTION PROCESSES USING *ZYMOMONAS MOBILIS*.

*Zymomonas mobilis* as ethanol producer has already been tested in a large variety of different continuous process.

#### 1.2.2.5.1 CELL RECYCLE STUDIES

Continuous fermentation with cell recycle exploits the ability of *Zymomonas mobilis* to produce alcohol at faster rate than a comparable biomass concentration of yeast therefore considerable effort has been made to maximise the potential of *Zymomonas mobilis* by developing arrangements to greatly increase cell densities with the fermenter. Cell recycle increases biomass density by recycling a proportion of cells in the outlet stream back into the fermenter.

Further advantages of cell recycle are: to enable chemostat to be operated at dilution rates greater than $\mu_{max}$, to able the chemostat to operate close to $D_m$, the dilution rate which gives maximum productivity without excessive unused substrate being left in the effluent. By recycling cells, a higher steady-state biomass concentration can be maintained which results in higher substrate comsumption.

Lee et al. (48) have reported a cell recycle system using millipore tangential flow microfiltration and a cell density of 40 g/l was obtained. When glucose was 12% (w/v) present in the medium, ethanol concentration was 60 g/l at dilution rate 3.3 $h^{-1}$. However due to frequent replacement of the membranes filters (every 12 - 15 hours) during the course of the experiments, it was concluded that this technique may be impractical with industrial substrates such as sugar cane juice and starch hydrolysates which could clog the membranes and certainly the capital costs and associated complexities involved with scheduling and replacement would largely offset any gains to be achieved in such a technique.

More recently, Rogers et al. (83) have reported the use of an ethanol-tolerant mutant of *Zymomonas mobilis* named ZM481 in cell recycle fermenters. The experiments were carried out with 18% and 20% (w/v) glucose media. Steady state conditions were only sustained at 18% glucose medium and biomass value was 33 g/l and an ethanol concentration of 90 g/l was achieved, at dilution rate of 0.95 $h^{-1}$.

The use of external sedimenter for cell recycle has been reported by de Bocks and van Eybergen (27). The use of a settler which is a simple and certainly cheaper method of recycling cells compared to filtration, was possible due to the flocculation characteristics of the strain used. The culture grew on 10% glucose (w/v) medium and growth limiting conditions were created by raising the temperature and lowering the amount of yeast extract. Under these conditions, the highest ethanol production of 40.5 $g.l^{-1}h^{-1}$ was achieved at dilution rate of 0.7 $h^{-1}$. Further increases in dilution rate gave lower ethanol values as well as decline in biomass concentration because of insufficient settling, wash out and insufficient biomass-growth in the medium used. Neverthless this method allowed for experiments of more than 6 weeks, without any cleaning of the cell recycle device.

Lee et al. (49) have studied the use of external cell recycle using 10% (w/v) glucose medium with *Zymomonas mobilis* ZM 401. The settler used was an inverted conical flask into which the overflow from the fermenter was introduced tangencially. At low dilution rates excellent flocculation was was achieved and ethanol concentration was

around 45 g/l. However above a dilution rate of 1.0 $h^{-1}$ active fermentation started to occur in the settler and cells were lost in the overflow stream.

1.2.2.5.2 CONTINUOUS UPFLOW FLOC (TOWER) FERMENTATION.

Attention has been drawn to the potential of continuous upflow fermenter for the accumulation of very high cell densities, and hence for the rapid conversion of sugars to ethanol.

Prince and Barford (77) have described the successful fermentation of glucose by *Zymomonas mobilis* using a tower fermenter. Cell densities up to 40 g/l were achieved with dilution rates of up to 2.3 $h^{-1}$ and a maximum productivity of 100g ethanol.$l^{-1}$.$h^{-1}$ with 98% conversion of the 10.5% glucose (w/v) feed was reported. It was pointed out that the limitation to performance with increase in throughtput arose from incomplete fermentation of the glucose feed, rather than washout of the flocculated bacteria.

The fermentation of sucrose by a flocculent isolate of *Zymomonas mobilis* in a continuous upflow reactor has been reported by Rodriguez and Callieri (81). The highest productivity of the system occurred when the dilution rate was almost 3 $h^{-1}$ with a 10% sucrose feed and 60% conversion to ethanol. Although it is mentioned that wash out did not occur, there was no measurement of biomass throughout the experiment.

## 1.3 AIMS

The general aims of this project were to acquire theoretical knowledge and practical experience relating to the use of continuous culture methodologies in fermentation.

The specific aims were to investigate the ethanolic fermentation of glucose, fructose and sucrose by a Brazilian isolate of *Zymomonas mobilis* (CP 4) using the following continuous culture methodologies:

(1) Classical chemostatic culture (including media evalution by delta pulsing)

(2) Continuous transient (square wave) culture.

# 2.MATERIALS AND METHODS

## 2.1 MICROORGANISM

*Zymomonas mobilis* CP4 was used throught in this study. This strain was originally isolated from fermented sugar cane juice (caldo de cana-picado) from Northeast Brazil by Gonçalves de Lima et al. (37). It has been suggested to group this strain, with other isolates, in one taxon, *Zymomonas mobilis* subsp. *mobilis* (96).

The bacteria are gram-negative rods, 2 to 6 by 1 to 2 µm, small and large cylinders, single or in pairs, rarely forming chains of a few cells. They are very motile.

The colonies are very typically mucoid. In liquid media, the bacteria develop abundantly with a flocculent deposit.

## 2.2 MAINTENANCE MEDIUM

| | |
|---|---|
| glucose | 2% (w/v) |
| Yeast extract | 1% (w/v) |
| $KH_2PO_4$ | 0.1% (w/v) |
| $(NH_4)_2SO_4$ | 0.1% (w/v) |
| $MgSO_4.7H_2O$ | 0.05% (w/v) |
| Agar | 2% (w/v). |

## 2.3 MAINTENANCE OF THE STRAIN

Subcultures were made from the original strain utilising the maintenance medium and incubated at 30° C for 72 hours.

The purity of the colonies was checked using Gram's stain. Then the new plates were kept at 4° C. Subcultures were made every 4 weeks.

## 2.4 BATCH CULTURE MEDIUM

For batch growth (including continuous culture start-up) the medium composition was the same as the maintenance one except agar was not included.

## 2.5 CONTINUOUS CULTURE MEDIA

For carbon limiting experiments (using glucose, fructose or sucrose) the medium composition was

| | |
|---|---|
| sugar (glucose, fructose, sucrose) | 2% (w/v) |
| Yeast extract | 0.5% (w/v) |
| $KH_2PO_4$ | 0.1% (w/v) |
| $(NH_4)_2SO_4$ | 0.1% (w/v) |
| $MgSO_4.7H_2O$ | 0.05% (w/v) |

For Nitrogen limitation the sugar concentrations was raised to 5% (w/v); all other components (including yeast extract) remained as above.

## 2.6 INOCULA

Inocula were prepared by transferring a few colonies from solid medium to Erlenmeyer flasks with 100 ml of maintenance medium followed by static incubation overnight at 35° C. This growth was used as inoculum for batch culture and continuous culture start-up.

## 2.7 MICROBIAL GROWTH SYSTEM

All continuous cultivation of cells and ethanol production were carried out in a 0.5 l laboratory fermenter (figure 2.7) with a 0.3 l working volume.

The temperature was maintained at 35° by the use of a 15 W heater (ceramic coated 10 Ohm resistance in borosilicate glass tube) controlled via an RS Temperature Controller (RS 344-625) using a Platinum Resistance Probe (RS 158-985) [RS Components P.O. Box 99 Corby,U.K.].

A magnetic stirrer was used to provide mild agitation.

The continuous addition of fresh medium was provided by means of an adjustable peristaltic pump and reactor contents left the vessel by an overflow pipe inserted through a side arm in the fermenter.

Continuous culture experiments were preceded by a batch and when the culture achieved the exponential phase, the medium flow was started.

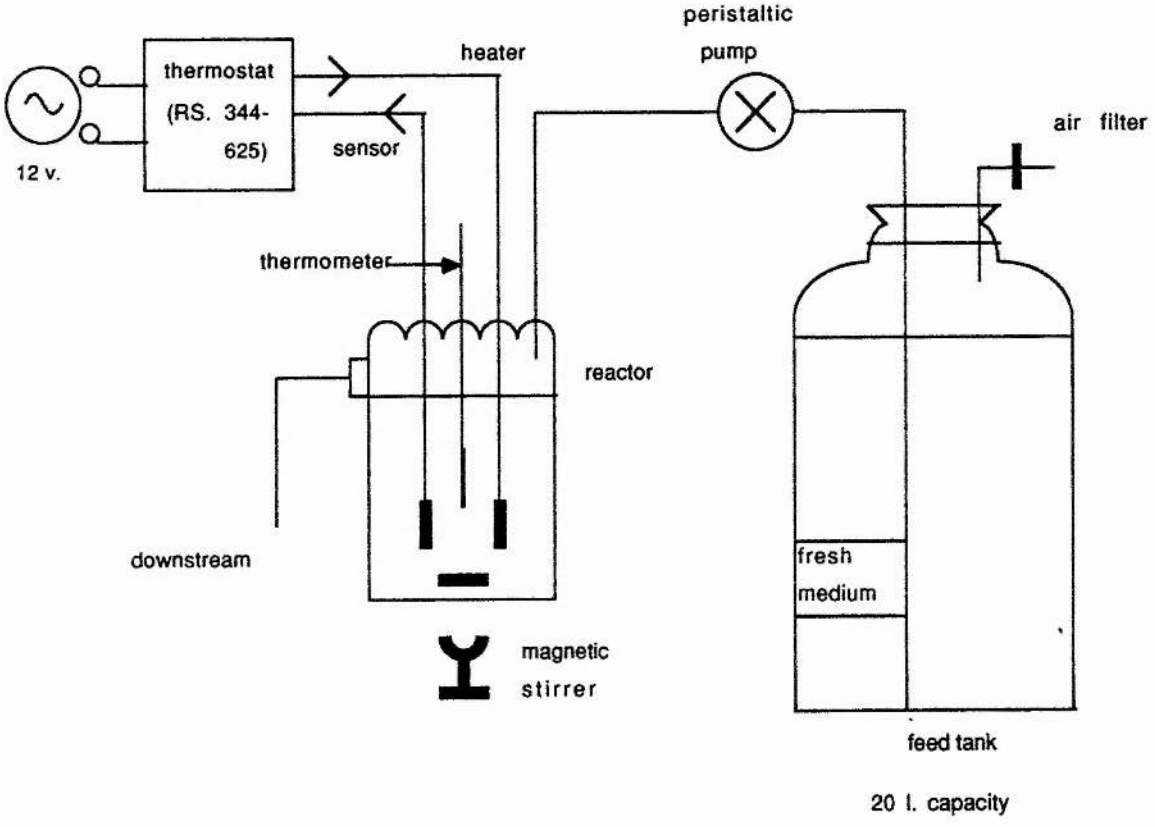



Fig.2.7 Experimental setup for continuous culture of Z.mobilis CP4.

The flow rate was checked out measuring the time (minutes) to fill a 20 ml volumetric flask with culture from the overflow pipe.

Sample collection was done from the overflow pipe with approximately 15 ml of culture being collected in a vial. Subsequently the material was divided into parts for the following analysis: biomass, sugar(s) and ethanol measurement.

## 2.8 MEASUREMENT OF CELL CONCENTRATION

The measurement of biomass was by measuring the absorption at 610 nm and the corresponding dry weight was obtained from a standard plot.

### 2.8.1. BIOMASS CONCENTRATION STANDARD PLOT

The standard curve for biomass concentration was constructed as follow:

*Zymomonas mobilis* CP4 was cultivated in maintenance medium in batch mode.

Biomass was harvested by centrifugation (1500 x g; 10 min.),washed and then resuspended to a completely homogeneous suspension in distilled water.

Subsequently, a series of suitable dilutions were made from the cell suspension and their absorbances recorded at 610 nm with a Pye SP 600 Series 2 (Unicam Instruments, Cambridge, England) spectrophotometer. Distilled water was used as the reference solution.

In the following stage, three accurately measured samples of the original cell suspension were reduced to dryness for 18 hours (using an oven 200° C) in weighing bottles of accurately known weight. Then, the average weight of the biomass in 1 ml samples was determined and lastly a graph of absorbance against dry weight of biomass per ml was constructed.

## 2.9 SUGAR MEASUREMENT.

Procedures for the estimation of glucose, fructose, sucrose and levan were carried out.

### 2.9.1 GLUCOSE AND FRUCTOSE.

The Somogyi and Nelson method (89) was used for measuring glucose and fructose when these sugars were individually used as carbon limiting in chemostat culture.

### 2.9.2 SUCROSE.

A procedure involving both the Somogyi and Nelson (89) and the glucose-oxidase (Sigma Chemical Co.Ltd.-Poole,U.K.) Methods for individual sugar concentrations was carried out when sucrose was the carbon source.

A flow sheet for analysis of fermentation liquor is given in figure 2.9.2.

Sucrose concentrations were calculated from the difference (A - B, see figure 2.9.2) between the total reducing sugars before and after HCl hydrolysis (51).

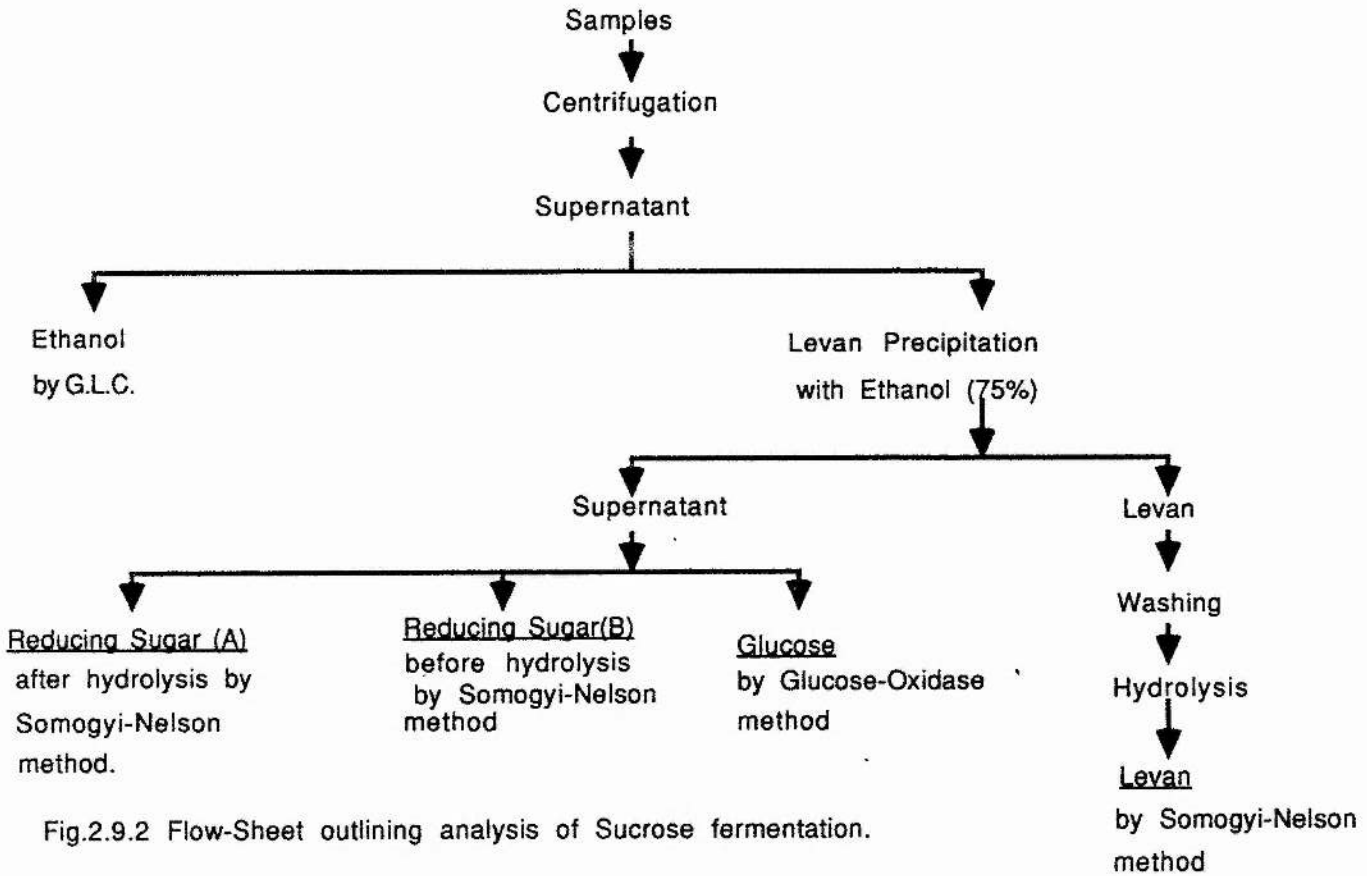



Fig.2.9.2 Flow-Sheet outlining analysis of Sucrose fermentation.

Fructose concentrations were estimated indirectly as the difference between the total reducing sugars before HCl hydrolysis (A, figure 2.9.2) and glucose-oxidase method.

Glucose was enzymatically determined using a kit based on the glucose oxidase-peroxidase system Sigma kit n° 510(SigmaChemical Co.Ltd.Poole,U.K.).

2.9.3 LEVAN.

Determination of levan as described by Dawes et al. (26) was as follow: 1 ml of fermentation broth is added to 3 ml ethanol 75% (v/v) in a centrifuge tube. A drop of 1% $CaCl_2$ is added to hasten flocculation. Subsenquently the suspension is centrifuged and the sedimented levan freed from reducing sugars and sucrose residues by twice-repeated solution in water over a water bath and re-precipitation with ethanol. After this, the final sediment is taken up in 3 ml 0.5% (v/v) oxalic acid and complete hydrolysis is effected by heating in a water- bath for 1 hour. The hydrolysate obtained is neutralized with 2 M NaOH, and then is diluted to a suitable volume with water. Finally, fructose in hydrolysates is estimated as reducing sugar by the Somogyi and Nelson Method (89).

2.10 MEASUREMENT OF ETHANOL

A gas-liquid chromatographic procedure was used to measure ethanol concentrations.

Measurements were made in a Pye Series 104 (Unicam Instruments, Cambridge,U.K.) gas chromatograph. The column was packed with Porapak Q (Waters Associates,

Inc.,Milford,Mass.USA) 80 - 100 mesh and maintained at 200° C during operation. The carrier gas was nitrogen at a flow rate of 37ml/min. The flame ionization detector was operated at 250° C.

Isopropanol was used as an internal standard. A series of aqueous standards were prepared containing ethanol in various concentrations from 1 to 9 mg/mg of isopropanol For each standard, the ethanol/isopropanol peak-height ratio was calculated and plotted against the respective concentration of ethanol

2.11 DELTA-TYPE OF PULSE PROCEDURE.

The pulse technique (68) as applied to a chemostat provides the flow system with a sudden change either stepwise, delta wise, or in frequency in any of the operational variables - limiting substrate concentration, dilution rate, temperature, pH value and so forth.

In this particular case a delta of pulse was used as a determined volume (typically 10 ml) of concentrated solution of glucose or yeast extract. The solution was added as a slug into the fermenter. The idea involved was to show whether glucose or yeast extract was the growth-limiting substrate or not.

Delta-type pulsing was performed as follows:

(i) A steady state was established, typically with a mid-range dilution rate of 0.2 $h^{-1}$.

(ii) A small slug of either glucose (to check for Carbon limitation) or Yeast Extract (to check for Nitrogen limitation) was quickly added (3 grams in 10 ml) by syringe directly into the fermenter reactor

## 2.12 CONTINUOUS PERIODIC OPERATION TECHNIQUE (13).

A number of experiments in this project dealt with continuous periodic operation technique sometimes simply referred as square wave

The continuous transient condition took the form of subjecting a steady state chemostat culture with *Zymomonas mobilis* at fixed value of dilution rate of 0.2 $h^{-1}$ to continuous and repetitive variations (square wave)in the input of glucose nutrient concentration.

The square waves in input nutrient concentration were achieved by connecting the chemostat to two nutrient reservoirs, each containing complete, defined medium, but with different concentrations of glucose as figure 2.12 (a) shows. At preset time intervals, a time system switched the nutrient feed between the two reservoirs. See figure 2.12 (b).

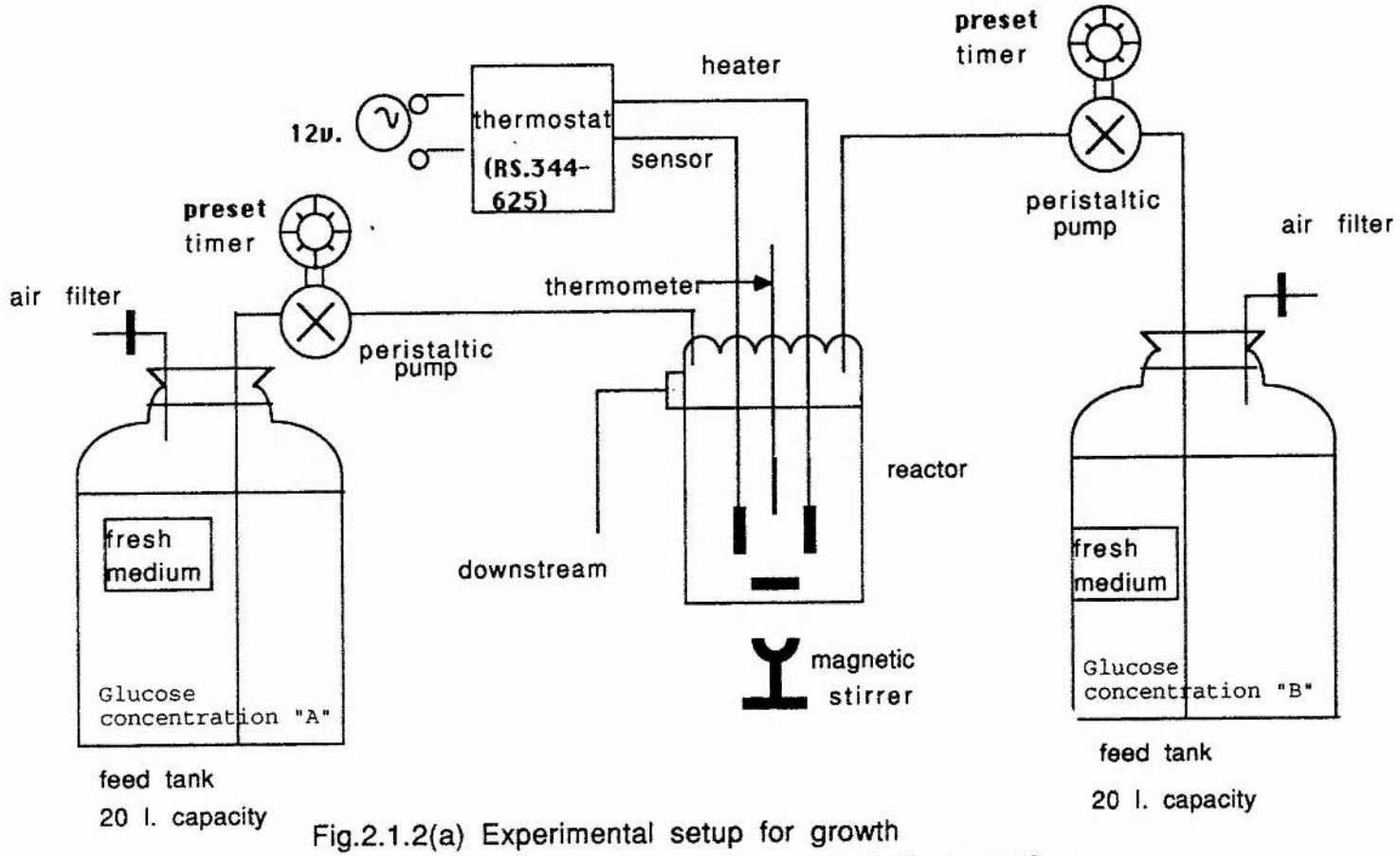



Fig.2.1.2(a) Experimental setup for growth of Z.mobilis CP4. under continuous periodic operation.

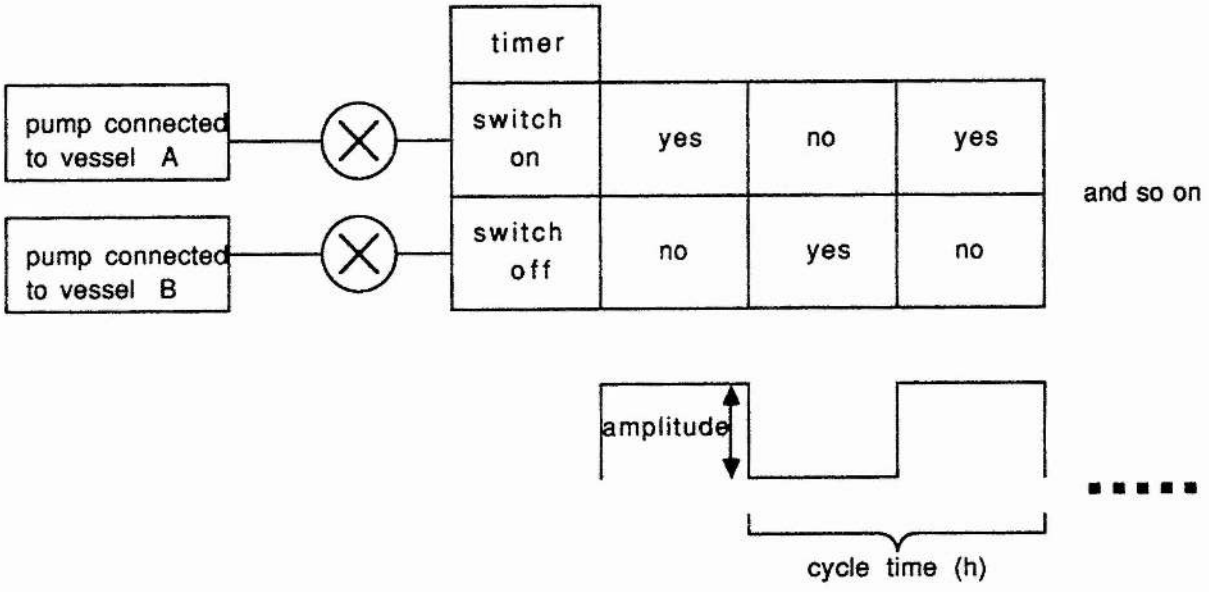



Figure 2.12 (b). Square waves in the incoming glucose concentration were achieved by connecting the bioreactor to two nutrient reservoirs, each containing the same medium but different glucose concentrations. At pre-established time intervals a timer/pump system switched the nutrient feed between both reservoirs, at constant dilution rate of 0.2 $h^{-1}$

## 2.13 CALCULATION OF THE MAXIMUM SPECIFIC GROWTH RATE.

The maximum specific growth rate denoted by the Greek letter $\mu$, was determined by two methods:

### 2.13.1 MAXIMUM SPECIFIC GROWTH RATE BY BATCH MODE EXPERIMENT.

The determination of the maximum specific growth rate by batch mode was calculated using the following equation:

$$\ln x = \ln x_o + \mu t \quad (75)$$

where :

$x_o$ = biomass (mg/ml) when t = 0

The plot of ln x against time in exponential growth will be a straight line. The slope is equal to $\mu$ .

### 2.13.2 MAXIMUM SPECIFIC GROWTH RATE BY WASHOUT METHOD.

By using the washout method of Pirt and Callow (76), it was also possible to estimate the maximum specific growth rate of *Zymomonas mobilis* CP4 grown in chemostat culture. The washout method determination of $\mu$ is based on obtaining the difference in slopes between the dilution rate and the washout of the culture.

The calculation of $\mu$ from the slope of the line observed is given in Appendix 7.

# 3. RESULTS AND DISCUSSION

## 3.1 BATCH CULTURE

The growth of *Zymomonas mobilis* CP4 in batch mode at 35° C batch culture using glucose at 2%(w/v) can be seen in figure 3.1.

The fig. 3.1 shows that the lag phase is short and difficult to be perceptible. Stanbury and Whitaker (90) have mentioned that in a commercial process the interest is to reduce the length of the lag phase as much as possible.

The occurrence of lag phase in a growth is caused by change in nutrition and physical environment. A change in a nutrient would probably involve the induction of one or more new enzymes and the time required to synthesize new enzyme(s) varies from minutes to hours.

Other two important aspects concerning the shortening of lag phase are: age of the cells of the inoculum and its size. The inoculum should use cells from logarithmic phase of growth, when the cells are still metabolically active and the size of it normally ranges between 3 and 10% of the culture volume (75).

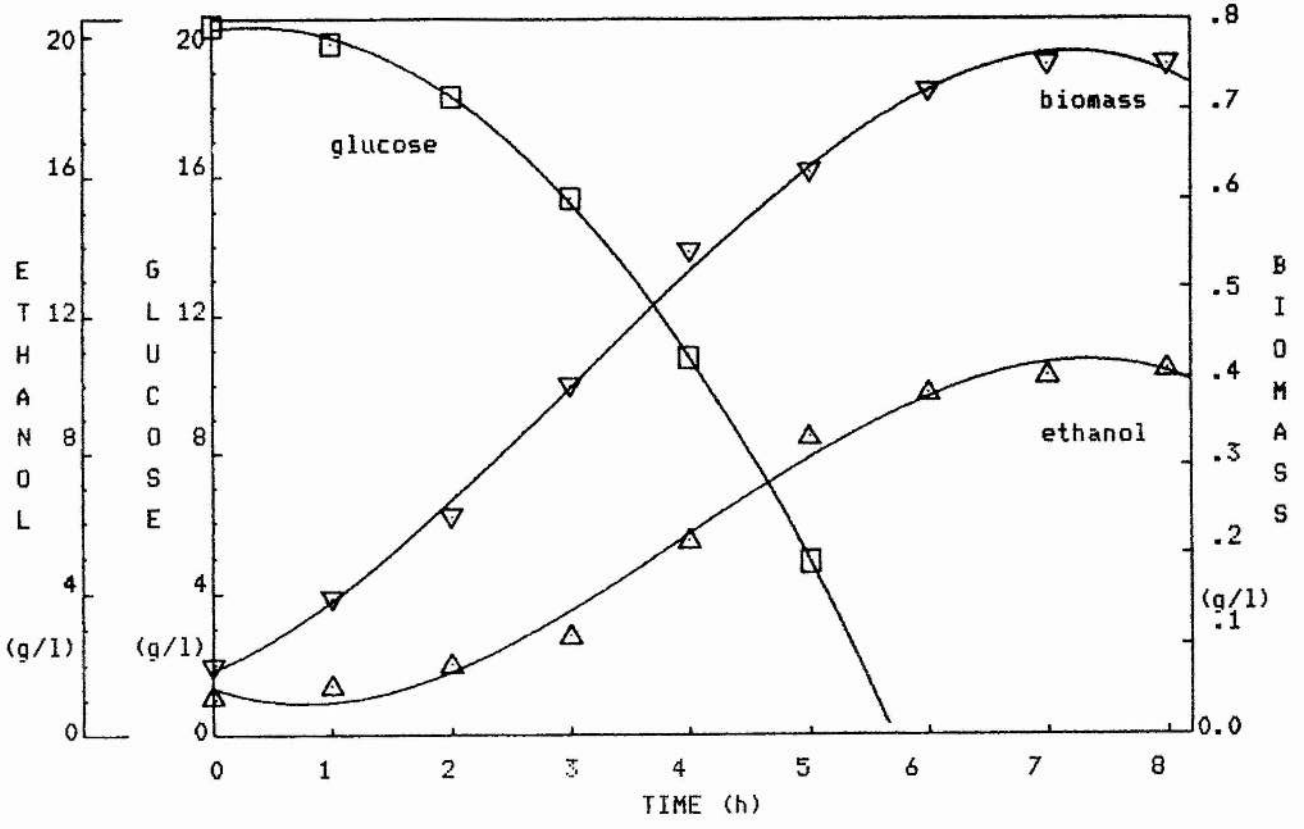



Fig. 3.1 Batch culture of Z. mobilis CP4 grown in 2%(w/v) glucose and 0.5%(w/v) yeast extract medium at 35°C.

3.1.1 ESTIMATION OF MAXIMUM SPECIFIC GROWTH RATE BY BATCH MODE

The figure 3.1.1 shows the plot of the natural logarithm of biomass concentration against time which during the exponential phase a straight line is obtained. The slope of the line gives a maximum specific growth rate ($\mu_{max}$) of 0.43 $h^{-1}$.(See section 2.13.1 and figure 3.1.1).

According to Monod (64), during the exponential phase of growth it is reasonable to consider that a steady state is established, where the relative concentrations of all metabolites and all the enzymes are constant. It is the only phase of growth when the properties of the cells may be considered constant. During this phase, the growth rate is also constant and equal to the maximum specific growth rate.

3.1.2 Value of Maximum Specific Growth Rate by Pirt and Callow Method.

Alternatively one can determine the maximum specific growth rate by a continuous flow method.

By using the washout method of Pirt and Callow, it was also possible to estimate the maximum specific growth of *Zymomonas mobilis* CP4 grown in chemostat culture.as figure 3.1.2 shows.

According to Pirt and Callow (76), when the growth in continuous culture is in accordance with the theory of Monod and Novick and Slizard and the dilution rate (D) is increased until the organism is flowing out of the culture faster than it is produced, that is D higher than $\mu_{max}$, an

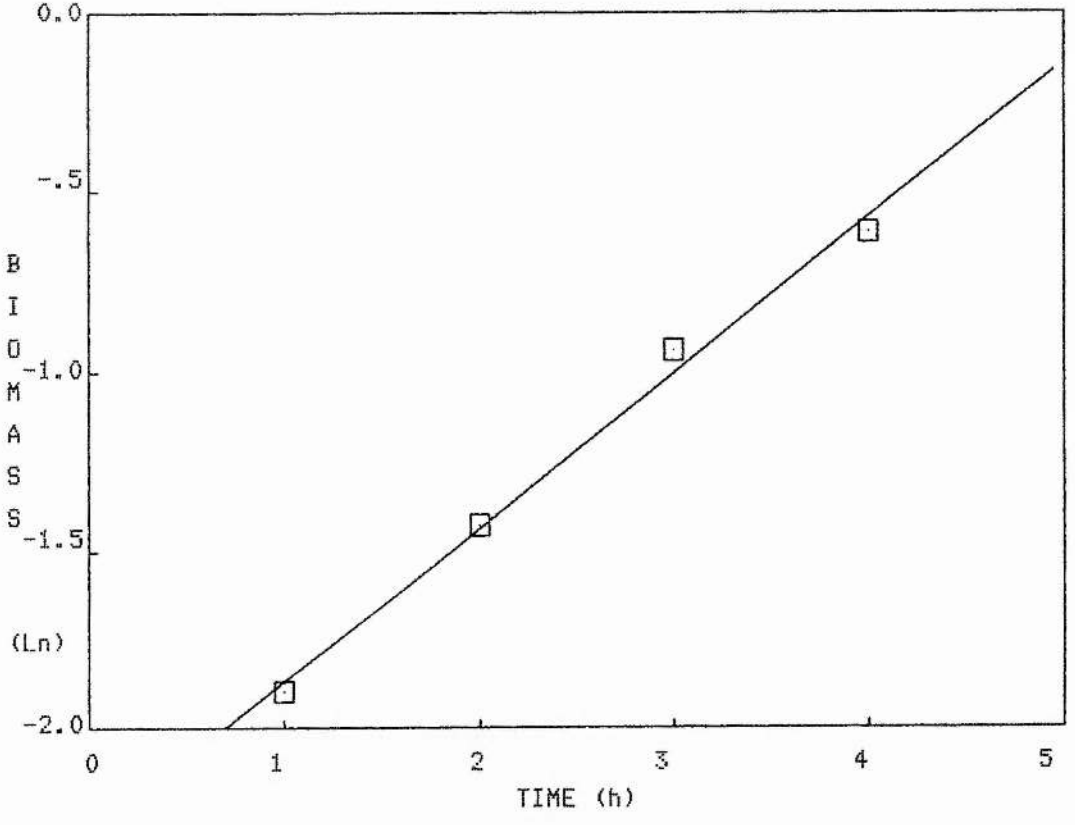



Fig.3.1.1 Values of biomass against time from exponential phase of batch culture of Z. mobilis CP4 grown in 2%(w/v) and 0.5%(w/v) yeast extract medium at 35 C.

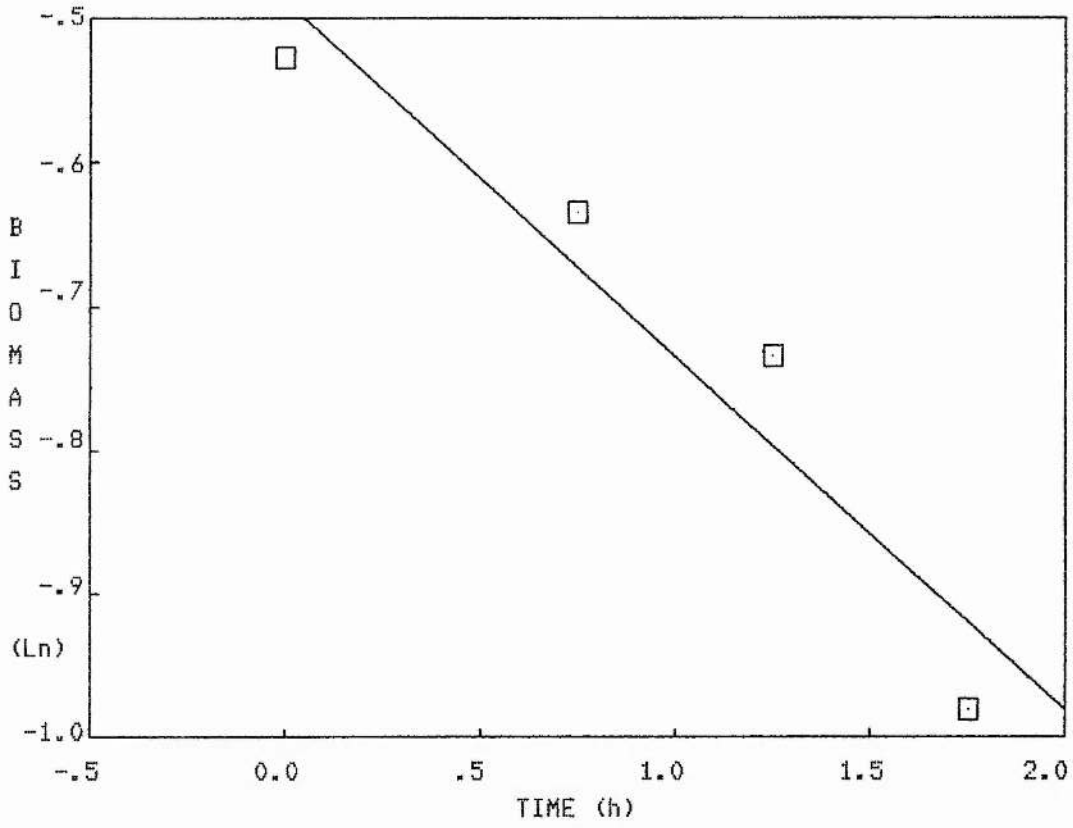



Fig. 3.1.2 Washout curve of Z. mobilis CP4 grown in chemostat culture in 2% (w/v) glucose and 0.5% (w/v) yeast extract medium at 35 C. Dilution rate changed at zero time from D= 0.22 /h to D= 0.61 /h.

excess of nutrients should be available and the organisms should grow at the maximum rate. Under this condition the logarithm of biomass plotted against the time should be a straight line with a negative slope equal to $\mu_{max}$ - D, and from this by inserting D one obtains $\mu_{max}$.

The time course of the experiment is shown in figure 3.1.2. The dilution rate of the culture in steady state was 0.22 $h^{-1}$ and that during the experiment 0.61 $h^{-1}$.

The calculation of $\mu_{max}$ from the slope of the line observed is given on appendix 7.

This dilution method gave a value of 0.37 $h^{-1}$ for $\mu_{max}$ which contrasts with the value of 0.43 $h^{-1}$ obtained from the batch mode curve of figure 3.1.2 However, the determination of $\mu_{max}$ by the Pirt and Callow Method is a more accurate one because it is carried out in a more constant environment

Kinetic data from batch growth in 2% (w/v) glucose are shown in table 3.1

Table 3.1. Batch culture of Z.mobilis CP4 grown in 2% glucose medium.

| $S_o$ | $S_c$ | $X_o$ | $X_t$ | $Y_{x/s}$ | $[P_f]$ | $[P]$ | $Y_{p/s}$ | $Q_s$ | $Q_p$ |
|---|---|---|---|---|---|---|---|---|---|
| g/l | g/l | g/l | g/l | g/g | g/l | g/l | g/g | g/g/h | g/g/h |
| 20.3 | 20.3 | 0.075 | 0.72 | 0.032 | 10.5 | 9.1 | 0.46 | 9.6 | 5.2 |

$S_o$ = initial substrate concentration

$S_c$ = amount of substrate consumed

$X_o$ = initial biomass concentration (inoculum)

$X_t$ = total biomass concentration

$Y_{x/s}$ = growth yield, g cells/g substrate used

$[P_f]$ = final ethanol concentration

$[P]$ = amount of ethanol formed

$Y_{p/s}$ = product yield g ethanol/g substrate used

$Q_s$ = specific rate of substrate uptake g substrate/ g cells/h

$Q_p$ = specific rate of ethanol formation, g ethanol/g cells/h

These observations show a good agreement when compared to the earlier work of Stenvensberg et al (93) who grew *Zymomonas mobilis* ATCC 29191 batchwise in a complex medium containing 2%(w/v) glucose at temperatures in the range of 30 to 36° C which revealed that the temperature optimum was 33° C with values of 0.53; 0.034 and 0.43 for cell growth, specific biomass production rate and ethanol productivity respectively. However, there are differences when values of $Q_s$ and $Q_p$ are compared but that is due to Stenvensborg et al.(128) having considered all phases of growth in their calculations while in this work it appeared more appropriate to consider only values taken from the exponential phase of growth.

This experiment was carried out at high temperature of 35° C and the value of biomass was low. This result and the values for biomass from Stevensborg et al.(93) support the results of Forrest (33) who has mentioned that a marked decrease in anabolism occurred at high temperatures with cells of *Zymomonas mobilis* grown at 1%(w/v) of glucose. Forrest has explained that tight coupling between anabolic and catabolic processes does not necessarily occur, so that in general the rate of energy production is not affected by the rate of energy utilization. It seems that such uncoupling can be affected by physical means. A high temperature is a case in point.

Senez (86) has suggested that the maximum efficiency of growth may be realized only over a restricted range of temperature, and that outside this range energetically uncoupled growth may take place even under conditions of

adequate nutrition, such that maximal coupling would be expected.

Lastly, the ethanol productivity value of 0.47 g/g is considered high since it represents 97% of the theoretical one.

## 3.2 CHEMOSTAT CULTURE

### 3.2.1 CHEMOSTATIC GROWTH UNDER CARBON LIMITATION

#### 3.2.1.1 GLUCOSE FERMENTATION

*Zymomonas mobilis* CP4 was grown in chemostat culture under glucose-limited conditions [$S_o$= 2% w/v] at 35° C and steady-states were obtained over a wide range of dilution rates, as shown in figure 3.2.1.1 and production parameters are given in table 3.2.1.1

The volumetric ethanol productivities can be compared with the results of Stevensborg et. al. (93), who grew *Z. mobilis* ATCC 29191 in chemostat culture, with 2% glucose. They obtained a volumetric ethanol productivity of 1.86 g/l/h when the value of dilution rate was 0.19 $h^{-1}$.

Productivity values increase with increasing dilution rates until it approximates the region where dilution rate exceeds the value of maximum growth rate. However, it should be noticed that high productivities are usually linked with drop in conversion rates (here represented by the value of conversion to ethanol efficiency achieved). Therefore it should be noticed that the two highest value of ethanol and biomass productivities correspond to those of glucose which are not being totally consumed and a portion of it were wasted in the wash out. Due to the fact that the substrate cost is estimated to represent at least 50 to 75% of the

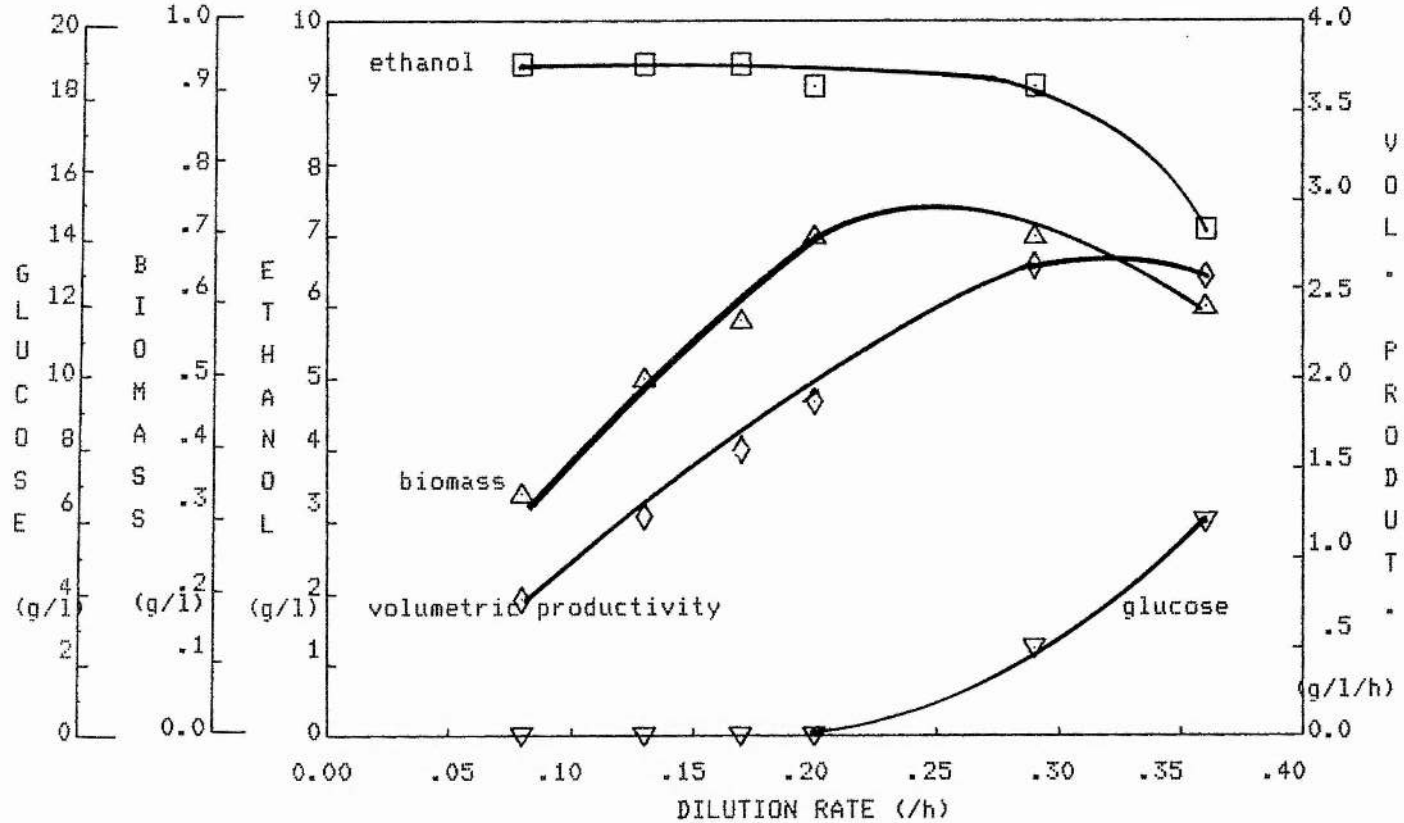



Fig. 3.2.1.1 Steady state data of continuous culture of Z. mobilis CP4. The culture was under glucose limitation of 2%(w/v) and 0.5%(w/v) yeast extract medium at temperature of 35 C.

Table 3.2.1.1 Continuous fermentation of glucose (19.6 g/l ) by Z.mobilis CP4 at different dilution rates.

| D ($h^{-1}$) | Sr (g/l) | X (g/l) | [P] (g/l) | V.P. (g/l/h) | $Y_{x/s}$ (g/g) | $Y_{p/s}$ (g/g) | Conversion Efficiency % |
|---|---|---|---|---|---|---|---|
| 0.08 | N.D. | 0.34 | 9.4 | 0.77 | 0.017 | 0.48 | 94 |
| 0.13 | N.D. | 0.5 | 9.4 | 1.23 | 0.026 | 0.48 | 94 |
| 0.17 | N.D. | 0.58 | 9.4 | 1.6 | 0.03 | 0.48 | 94 |
| 0.20 | N.D. | 0.7 | 9.4 | 1.87 | 0.036 | 0.48 | 94 |
| 0.29 | 2.5 | 0.7 | 9.1 | 2.63 | 0.041 | 0.46 | 91 |
| 0.36 | 6 | 0.6 | 7.1 | 2.57 | 0.044 | 0.36 | 71 |

[P] = ethanol concentration

$S_r$ = residual substrate

N.D. = Not Detectable

V.P. = volumetric productivity

X = biomass concentration

D = dilution rate

$Y_{p/s}$ = product yield g ethanol/g substrate used

$Y_{x/s}$ = growth yield, g cells/g substrate used

$$\text{conversion efficiency} = \frac{\text{ethanol concentration}}{0.51 \times \text{substrate concentration}} \times 100 \ ;$$

assuming that glucose (180) ⟶ 2 ethanol (92) + 2 carbon dioxide (88)

total production cost of ethanol, fermenters are normally run at maximal conversion rate and not at maximal productivity (97).

### 3.2.1.2 FRUCTOSE FERMENTATION

*Zymomonas mobilis* CP4 was cultivated in continuous culture on medium containing 20 g/l of fructose. Steady states data for biomass, ethanol and residual fructose concentrations are shown in figure 3.2.1.2 and table 3.2.1.2

Maximal values of biomass and ethanol were respectively 0.37 g/l and 9.2 g/l at dilution rate 0.16 $h^{-1}$. Biomass and ethanol yields were 0.019 and 0.48 g/g/ respectively. The maximum volumetric productivity of 1.8 g/l.h was observed at dilution rate 0.21 $h^{-1}$ but the value of conversion to ethanol efficiency started falling at that same value of dilution rate.

The value of biomass produced was low and this observation has been noticed by other authors. Dawes et al.(26) and McGill and Dawes (59) have previously reported relatively low biomass yield and slow growth on fructose. More recently, significant differences were observed with growth rate and cell yield by Rogers et al. (81). Batch fermentations utilised high concentrations (250 g/l) of different substrates and it was found that fructose fermentation produced about one-half of biomass compared with those carried out on glucose or sucrose. According to Viikari and Korhola (107) during the metabolism of fructose

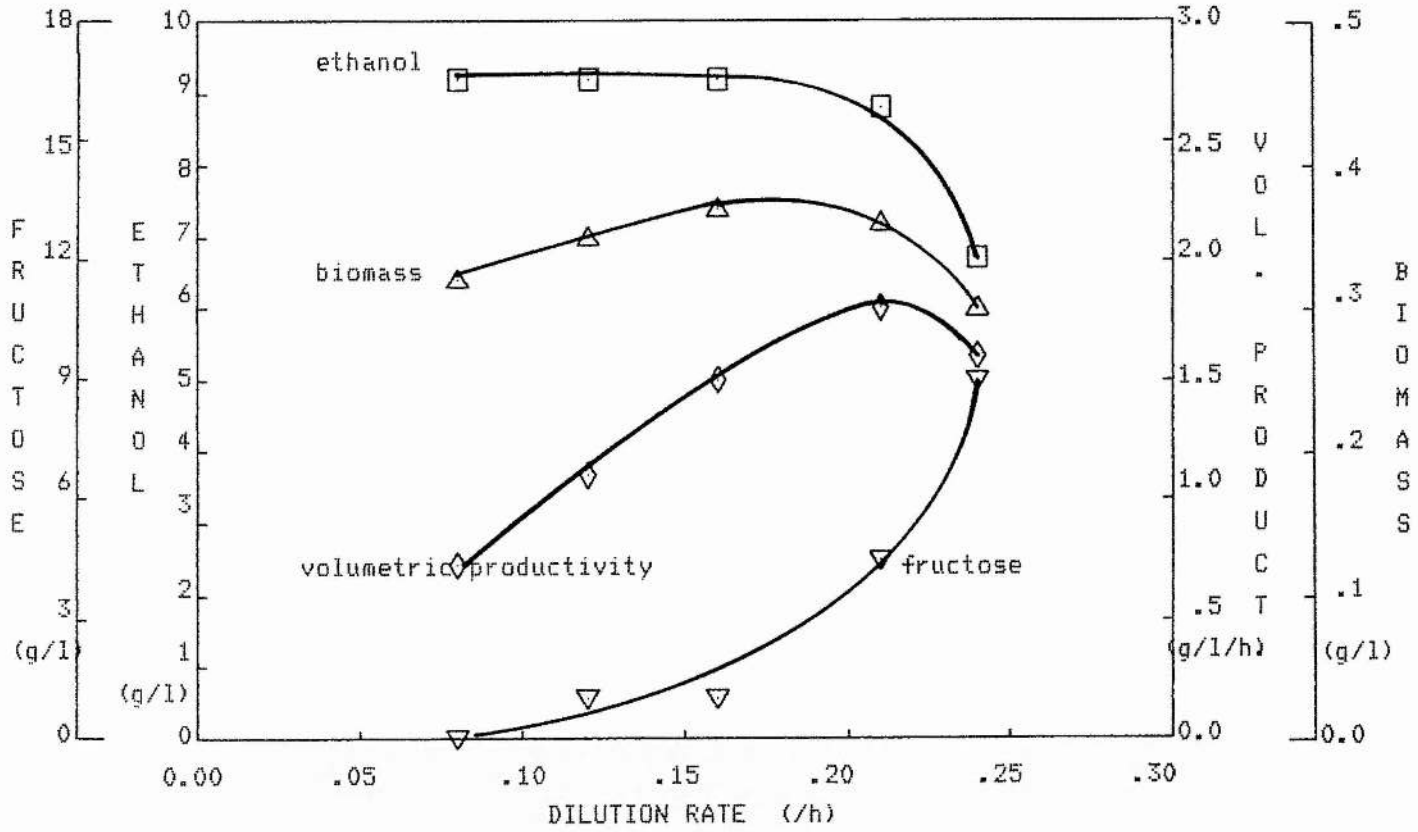



Fig. 3.2.1.2 Steady state data of continuous culture of Z. mobilis CP4. The culture was under fructose limitation of 2%(w/v) and 0.5%(w/v) yeast extract medium at temperature of 35 C.

Table 3.2.1.2 Continuous fermentation of fructose (20 g/l ) by Z.mobilis CP4 at different dilution rates.

| D | Sr | X | [P] | V.P. | $Y_{x/s}$ | $Y_{p/s}$ | Conversion Efficiency |
|---|---|---|---|---|---|---|---|
| ($h^{-1}$) | (g/l) | (g/l) | (g/l) | (g/l/h) | (g/g) | (g/g) | % |
| 0.08 | N.D. | 0.32 | 9.2 | 0.73 | 0.016 | 0.46 | 90 |
| 0.12 | 1 | 0.35 | 9.2 | 1.1 | 0.018 | 0.46 | 90 |
| 0.16 | 1 | 0.37 | 9.2 | 1.5 | 0.019 | 0.46 | 90 |
| 0.21 | 4.5 | 0.36 | 8.8 | 1.8 | 0.023 | 0.44 | 86.3 |
| 0.24 | 9 | 0.3 | 6.7 | 1.6 | 0.027 | 0.34 | 66 |

N.D.: Not Detectable

the relatively low cell yield can partly be explained by the loss of ATP in the formation of dihydroxyacetone and glycerol and partly by the toxic effect of dihydroxyacetone and acetaldehyde on the growth of the organism.

Continuous fermentation of fructose carried out in this work showed that volumetric productivities reached its maximum value when dilution rate was around 0.2 $h^{-1}$. Toran-Diaz et al. (102) have reported continuous fermentation using fructose at 10%(w/v) in the medium. A superior value of volumetric productivity of 3 g/l.h was observed at dilution rate around 0.1 $h^{-1}$.

When fructose is the substrate, high values of dilution rate have been only possible by the use of modification in the fermenter such as internal settler (102) and the utilization of a flocculent strain (3).

### 3.2.1.3 SUCROSE FERMENTATION

*Zymomonas mobilis* CP4 was used for ethanol production from sucrose at 21.1 g/l in chemostat culture. The kinetic parameters of the fermentation at different dilution rates are presented in tables 3.2.1.2(a) and 3.2.1.3(b) and figure 3.2.1.3.

Sucrose was extensively hydrolysed in all values of dilution rate. Only low amounts of sucrose remained unhydrolysed, at high dilution rates. Viikari and Linko (108) have mentioned that even at high substrate concentrations the hydrolysis of sucrose was in no case a limiting factor of the fermentation.

Fructose and, to a lesser extent glucose were present in the fermenter at increasing concentrations in the highest dilution rates tested. There was an increase in levan formation when dilution rate exceeded 0.2 $h^{-1}$ and after it remained constant.

Except for dilution rate of 0.35 $h^{-1}$, the ethanol yield was constant (0.4 g/g) in the whole range of dilution rates which represents around 80 % of conversion to ethanol efficiency. That is a low value compared with those for glucose and fructose. According to Viikari (104) the ethanol yield from the fermentation of sucrose by *Zymomonas mobilis* strains is low because of formation of levan and sorbitol. The former is connected with hydrolysis of sucrose and the

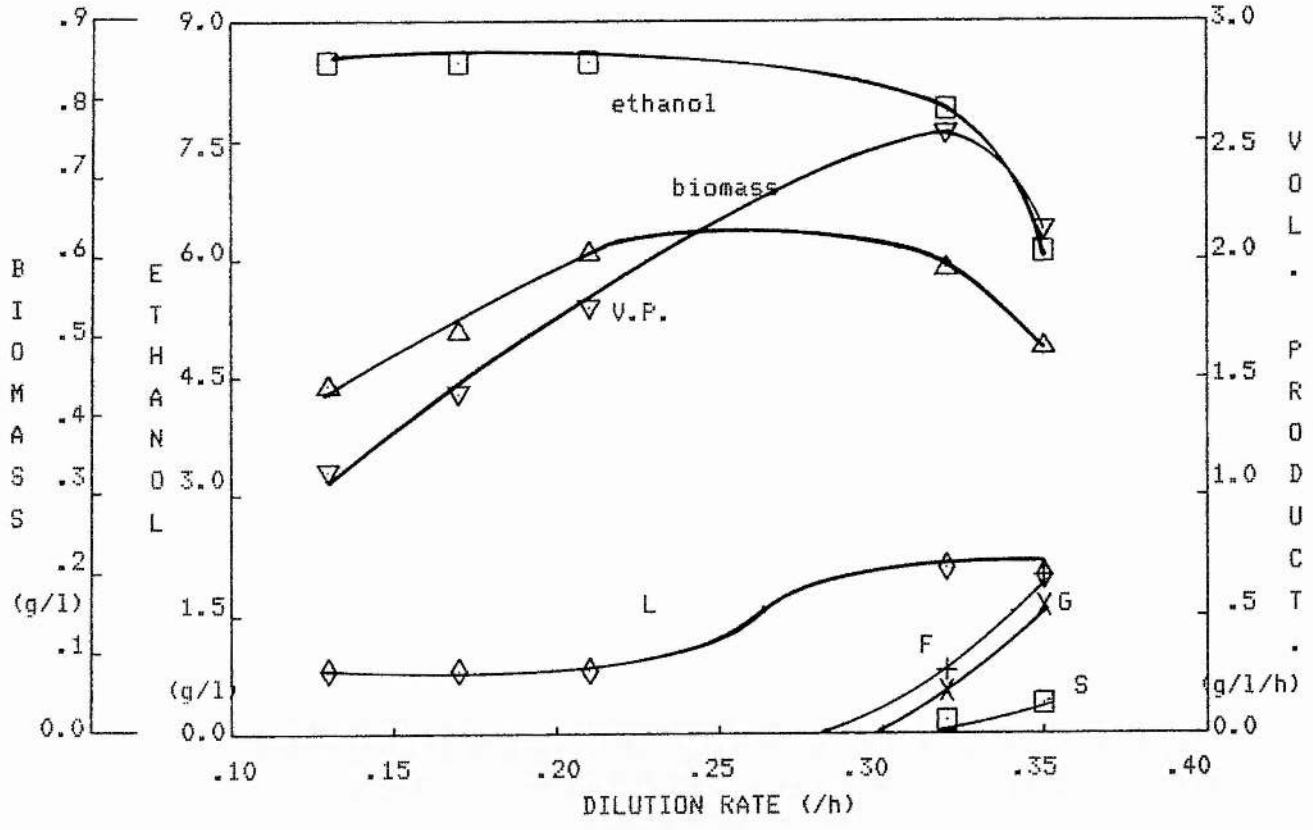



Fig. 3.2.1.3 Steady state data of continuous culture of Z. mobilis CP4. The culture was under sucrose limitation of 2%(w/v) and 0.5%(w/v) yeast extract medium at temperature of 35°C. The unhydrolysed sucrose (S) and by-products levan (L), fructose (F) and glucose (G) utilise the scale of ethanol.

Table 3.2.1.3(a) Continuous fermentation of Sucrose (21,1 g/l ) by Z.mobilis CP4 at different dilution rates.

| D | X | [P] | V.P. | $Y_{x/s}$ | $Y_{p/s}$ | Conversion Efficiency |
|---|---|---|---|---|---|---|
| ($h^{-1}$) | (g/l) | (g/l) | (g/l/h) | (g/g) | (g/g) | % |
| 0.13 | 0.44 | 8.5 | 1.1 | 0.021 | 0.4 | 79 |
| 0.17 | 0.51 | 8.5 | 1.43 | 0.024 | 0.4 | 79 |
| 0.21 | 0.61 | 8.5 | 1.8 | 0.029 | 0.4 | 79 |
| 0.32 | 0.59 | 7.9 | 2.53 | 0.03 | 0.37 | 73 |
| 0.35 | 0.49 | 6.1 | 2.13 | 0.029 | 0.29 | 57 |

Table 3.2.1.3(b) Unhydrolysed sucrose and by-product formation during fermentation of sucrose (21,1 g/l) by Z.mobilis CP4 at different dilution rates.

| D ($h^{-1}$) | T.R.S.* (g/l) | Sucrose (g/l) | Fructose (g/l) | Glucose (g/l) | Levan (g/l) |
|---|---|---|---|---|---|
| 0.13 | N.D. | N.D. | N.D. | N.D. | 0.8 |
| 0.17 | N.D. | N.D. | N.D. | N.D. | 0.8 |
| 0.21 | N.D. | N.D. | N.D. | N.D. | 0.8 |
| 0.32 | 1.5 | 0.16 | 0.8 | 0.54 | 2.1 |
| 0.35 | 4 | 0.38 | 2 | 1.62 | 2 |

* Total Reducing Sugar N.D.: Not Detectable

latter with ethanol formation. In addition to high molecular weight levan (precipitable form), fructo-oligmers have also been detected by Barrow et.al.(12). Here only the precipitable form was measured. If there was fructo-oligmers production, they were measured as fructose. However, this seems unlikely considering the small amount of fructose found in the samples.

### 3.2.1.4 MEASUREMENT OF MAINTENANCE ENERGY COEFFICIENT

By extrapolating $Q_s$ to zero dilution rate (figure 3.2.1.4), the maintenance energy coefficient, denoted by the letter "m" can be calculated as 1.1 $h^{-1}$ for glucose, 1.4 $h^{-1}$ for fructose and 0.5 $h^{-1}$ for sucrose, from the following equation proposed by Pirt (75).

$$Q_s = \mu/Y + m$$

The concept of maintenance energy can be traced deeply into the past. It was first mentioned by Terroine and Wurmser in 1922 (99) and more recently Herbert in 1958 (39) to explain the deviation in chemostat cultures from the Monod theory.

Plotting the specific substrate utilisation rate ($Q_s$) against dilution rate yields a straight line which cuts the $Q_s$ axis at a positive value. Thus, at zero dilution rate, i.e. when there is no growth, there is a residual substrate demand.

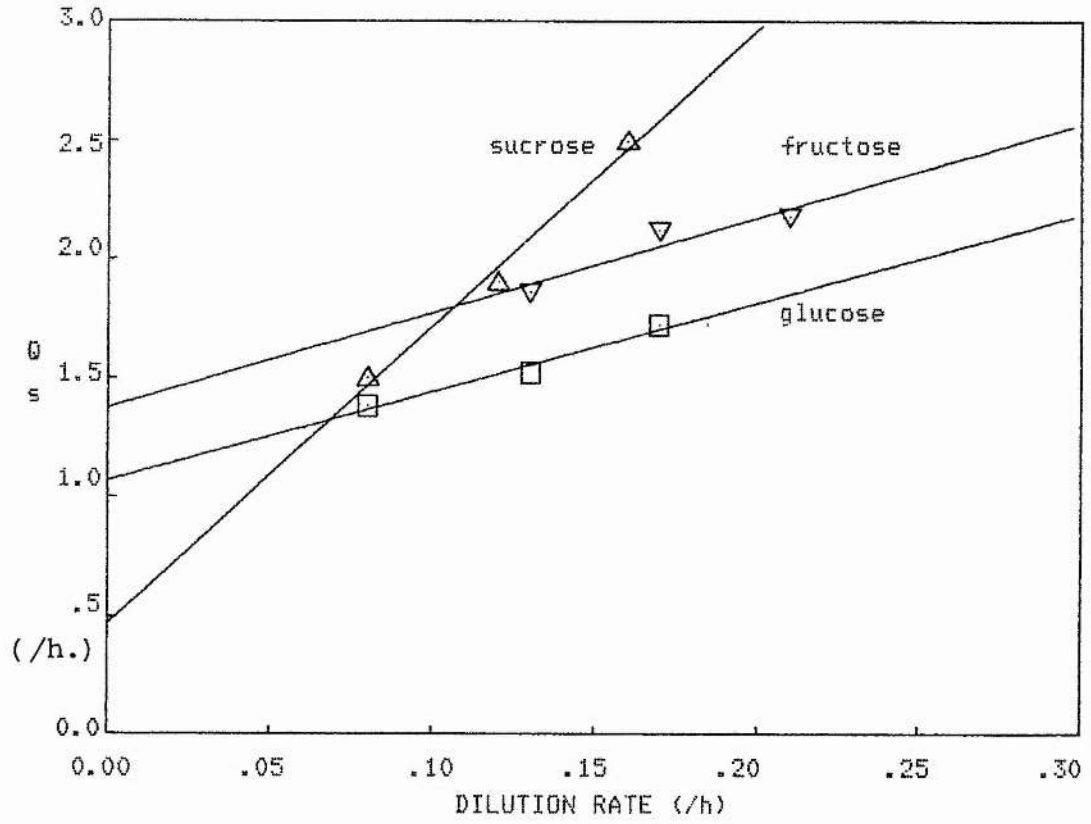



Fig. 3.2.1.4 Values determination of maintenance energy coefficient for glucose, fructose and sucrose.

According to Pirt (75) energy requirements for maintenance purposes are turnover of cell materials, osmotic work to maintain concentrations gradients between the cell and its exterior, and cell motility.

The values of maintenance energy coefficient calculated here can be compared with a previous investigation by Fieschko and Humphrey (32). Working with *Zymomonas mobilis* ATCC 10988 in a defined salts medium (containing 2% glucose, with ammonium as nitrogen source and 1,2 x $10^{-5}$ g/l pantothenate) these investigators found that elevating the temperature caused an increase in the maintenance energy coefficient from 0.5 - 2.2 g glucose/g cell/h.

The value of maintenance energy coefficient is expected to vary according to Goma et al.(36). These authors have mentioned that in addition to those factors pointed out by Pirt which influence the value of maintenance energy coefficient others should be also taken into account such as the factor representing the part of the substrate used for the formation of product and secondary metabolites. According to them the factors involved in maintenance energy are numerous and complex and it is rather doubtful a constant character for it since all those factors can change with time or with the specific growth rate.

The different values found for maintenance energy coefficient in this work may suggest that variations in the carbon source constitute factors which influence its value.

### 3.2.1.5 CONVERSION TO ETHANOL EFFICIENCY

The figure 3.2.1.5 represents the different values of conversion to ethanol efficiency for each individual sugar fermentation at 2% (w/v) in the range of dilution rate tested.

Conversion efficiency varied from 80% to 97% for dilution rate up to 0.2 $h^{-1}$. At that range of dilution rates glucose showed the highest values (around 95%), followed by fructose (90%) and then by sucrose (80%). However, there was a fall in conversion efficiency at dilution rates higher than 0.2 $h^{-1}$. Fructose had its value down to 67% at dilution rate of 0.24 $h^{-1}$. Sucrose conversion to ethanol efficiency fell to values of 76 and 58% at dilution rates of 0.32 and 0.35 $h^{-1}$ respectively while glucose conversion efficiency was still high at dilution rate of 0.29 $h^{-1}$ and dropped to 72% at dilution rate of 0.36$h^{-1}$.

Thus, due to these results, the further experimentation with chemostat utilised glucose at higher concentration under the condition of nitrogen limitation.

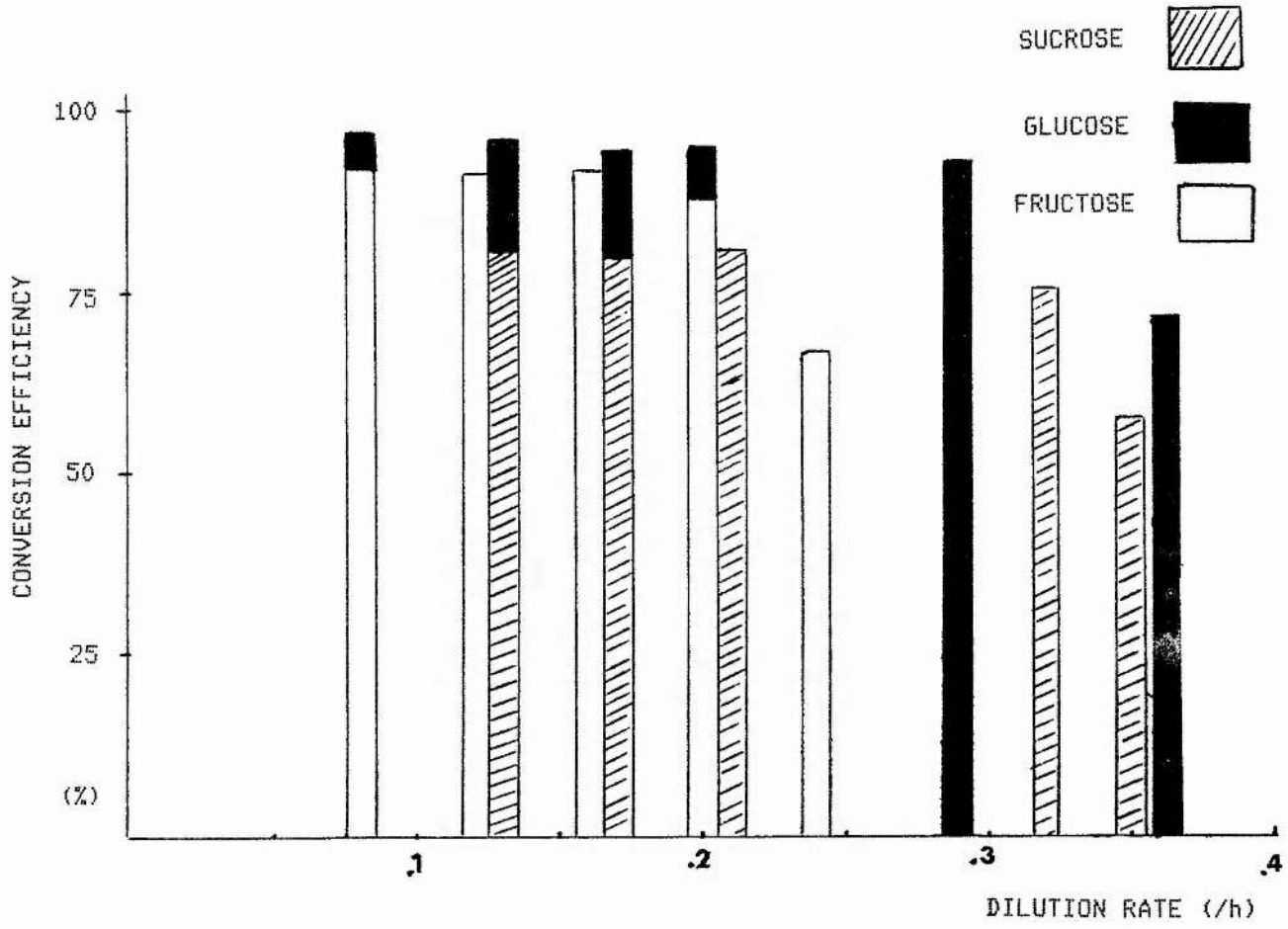



Fig. 3.2.1.5 Conversion efficiency to ethanol for glucose, fructose and sucrose at different dilu tion rate values.

### 3.2.2 CHEMOSTAT GROWTH UNDER NITROGEN LIMITATION

*Zymomonas mobilis* CP4 was studied in chemostat culture under nitrogen limited conditions at 35° C. This condition was achieved by increasing the concentration of glucose to 5% (w/v) while the nitrogen source, yeast extract remained 0.5% (w/v) in the medium.

Steady state at dilution rate of 0.17 $h^{-1}$ following change from batch to continuous culture is shown in figure 3.2.2 and values in table 3.2.2

A short period of oscillations was observed following commencement of continuous culture before the establishiment of steady state. Lee et al.(53,52) and Bruce et al.(18) have reported typical oscillations in biomass, glucose and ethanol with *Zymomonas mobilis* ATCC 10988 and ZM4 with 15% glucose medium following a change in dilution rate and commencement of continuous cultue.

Under this condition of nitrogen limiting and value of dilution rate, the steady state conditions is inefficient as glucose was not fully metabolised . Rates representing around 25% of feed medium glucose were being wasted while the value of conversion to ethanol efficiency was around 60%.

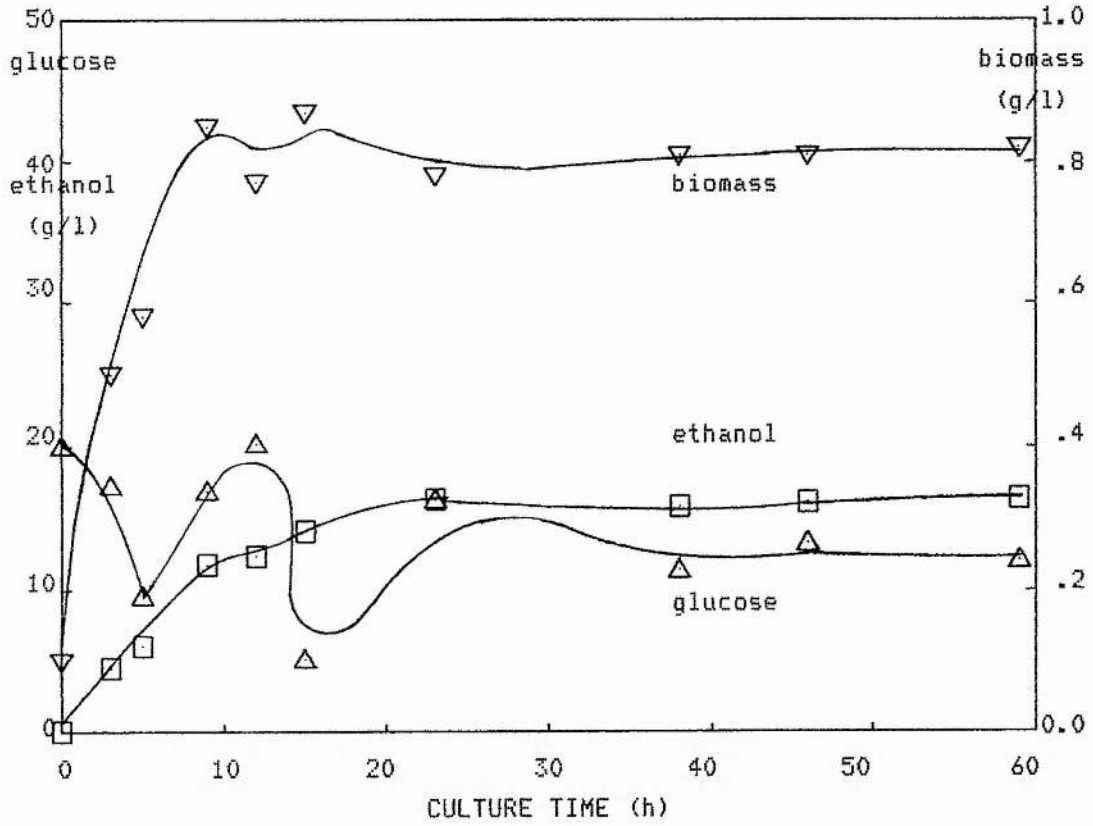



Fig. 3.2.2 Fermentation of glucose at 5%(w/v) under nitrogen limited condition by yeast extract at 0.5%(w/v) at 35 C. Oscillations are shown following change from batchwise (first 5 hours of culture) to chemostat at fixed dilution rate 0.17 /h.

Table 3.2.2 Fermentation of glucose by Z.mobilis CP4 under nitrogen limitation condition. Data refer to batchwise and chemostat culture. (dilution rate=0.17 h )

| | culture time (h) | X (g/l) | Sr (g/l) | [P] (g/l) |
|---|---|---|---|---|
| Batchwise | 3 | 0.5 | 17.3 | 4.5 |
| | 5 | 0.58 | 9.6 | 6.1 |
| Chemostat | 9 | 0.85 | 16.9 | 11.8 |
| | 12 | 0.77 | 20.2 | 12.4 |
| | 15 | 0.87 | 5.1 | 14.1 |
| | 23 | 0.78 | 16.3 | 16.3 |
| | 38 | 0.81 | 11.4 | 15.8 |
| | 46 | 0.81 | 13.3 | 16.1 |
| | 59 | 0.82 | 12.1 | 16.4 |

### 3.2.2.1 DELTA-TYPE OF PULSE

It was decided to check the system in respect of rate-limiting nutrients carrying out pulsing of single components of the medium in the chemostat. This technique represents a very efficient tool for detailed nutrients studies. Glucose and yeast extract were the components to be tested. The former, mainly to ensure that an extra pulse of glucose into the system would result in no increase in biomass or ethanol concentration clearly showing that the system was not under carbon limitation.

There are only few publications concerning the pulse method. Nagai et al.(68) have published the *Azotobacter vinelandii* response to a delta type of pulse effected in a medium via glucose. According to them, the fraction of RNA in the cells increased sigmoidally as the protein fraction decreased exponentially while other cellular components remained approximately unchanged. Harvey (38) has reported the use of pulse method on glucose-limited chemostat of *Escherichia coli.* Supplementation with glucose plus 20 amino acids, produced an immediate increase in the specific rate of biomass and RNA synthesis followed by an increase in the specific rate of protein synthesis.

The figure 3.2.2.1(a) and table 3.2.2.1(a) and figure 3.2.2.1(b) and table 3.2.2.1(b) respectively ilustrate pulses carried out with glucose and yeast extract in nitrogen-limited chemostat culture of *Zymomonas mobilis* CP4. When they are compared, the effects on biomass, ethanol and

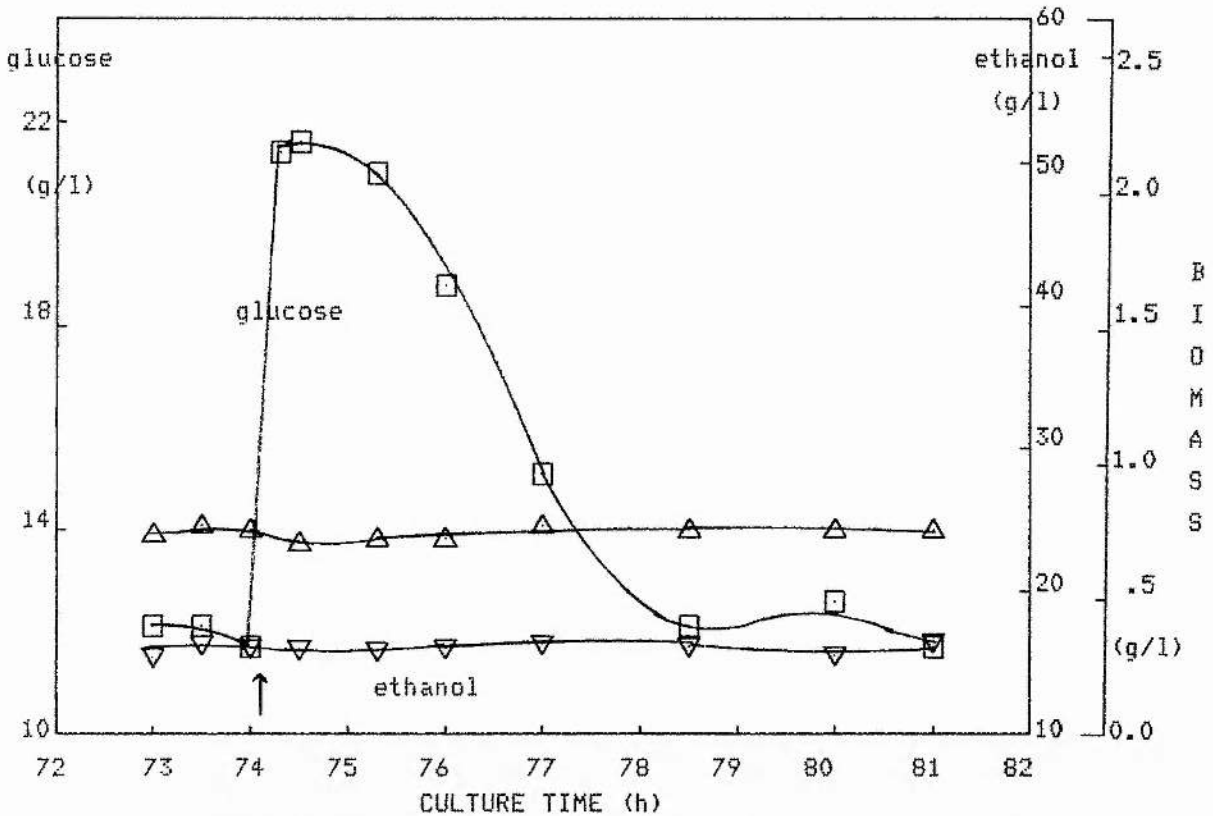



Fig. 3.2.2.1(a) Glucose pulsing in Nitrogen-limited chemostat culture of Z. mobilis CP4 during steady state. The cells were grown in 5%(w/v) glucose and 0.5%(w/v) yeast extract medium at 35 C and D= 0.2/h The arrow indicates when the pulse was carried out.

Table 3.2.2.1(a)Values of residual glucose, biomass and ethanol for a chemostat culture of Z.mobilis CP4 subjected to a delta pulse of glucose.

| culture time (h) | Sr (g/l) | X (g/l) | [P] (g/l) |
|---|---|---|---|
| 73 | 12.1 | 0.8 | 15.4 |
| 73.5 | 12.1 | 0.82 | 16.2 |
| 74 | 11.7 | 0.81 | 16 |
| 74.3 | 21.4 | | |
| 74.5 | 21.6 | 0.78 | 15.9 |
| 75.3 | 21 | 0.79 | 15.8 |
| 76 | 18.8 | 0.79 | 16 |
| 77 | 15.1 | 0.82 | 16.3 |
| 78.5 | 12.1 | 0.81 | 16.1 |
| 80 | 12.6 | 0.81 | 15.5 |
| 81 | 11.7 | 0.8 | 16.4 |

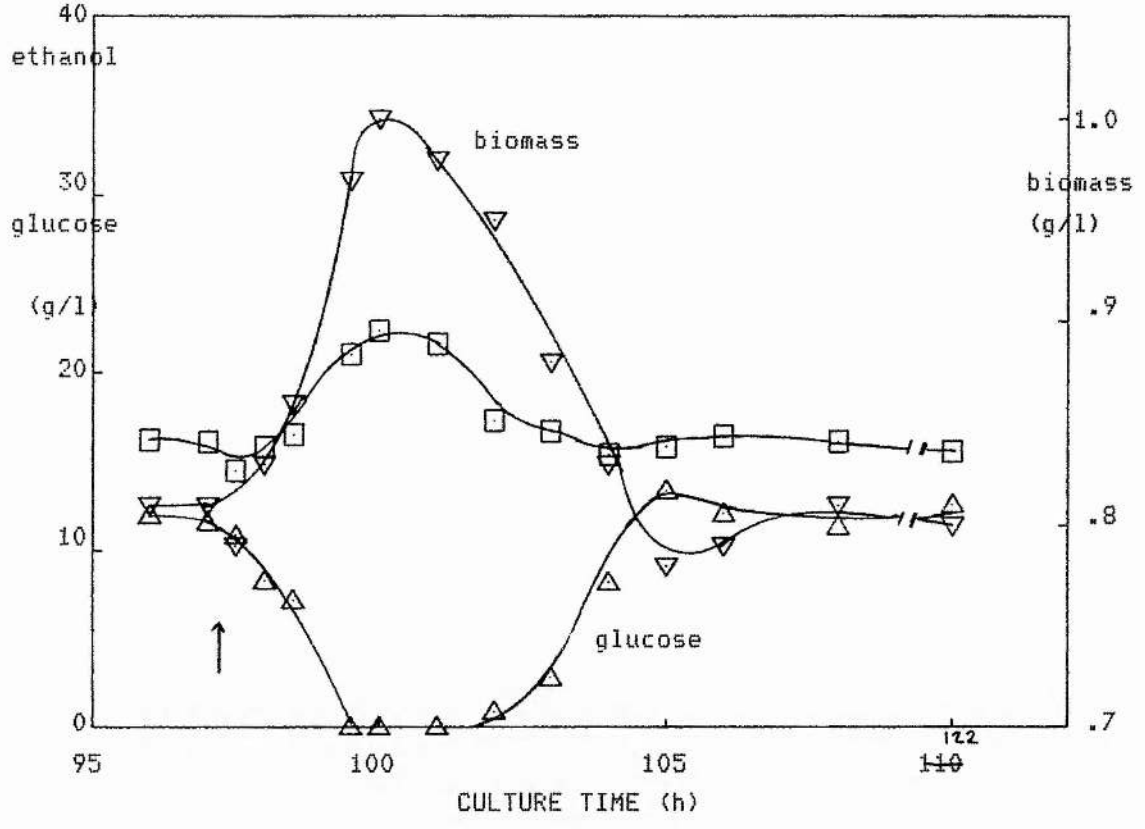



Fig. 3.2.2.1(b) Yeast extract pulsing in Nitrogen-limited chemostat culture of Z. mobilis CP4 during steady state. The cells were grown in 5%(w/v) glucose and 0.5%(w/v) yeast extract medium at 35 C and D= 0.2 /h. The arrow indicates when the pulse was carried out.

Table 3.2.2.1(b) Values of residual glucose, biomass and ethanol for a chemostat culture of Z.mobilis CP4 subjected to a delta pulse of yeast extract.

| culture time (h) | Sr (g/l) | X (g/l) | [P] (g/l) |
|---|---|---|---|
| 96 | 12 | 0.81 | 16.2 |
| 97 | 11.6 | 0.81 | 16.1 |
| 97.5 | 10.9 | 0.79 | 14.5 |
| 98 | 8.3 | 0.83 | 15.8 |
| 98.5 | 7.2 | 0.86 | 16.5 |
| 99.5 | N.D. | 0.97 | 21 |
| 100 | N.D. | 1 | 22.3 |
| 101 | N.D. | 0.98 | 21.6 |
| 102 | 0.9 | 0.95 | 17.3 |
| 103 | 2.8 | 0.88 | 16.7 |
| 104 | 8.2 | 0.83 | 15.4 |
| 105 | 13.4 | 0.78 | 15.8 |
| 106 | 12.1 | 0.79 | 16.4 |
| 108 | 11.4 | 0.81 | 16.1 |
| 122 | 12.6 | 0.80 | 15.6 |

N.D.: Not Detectable

glucose consumption were rather different. When glucose was used in the pulse, it was washed out of the chemostat and no change in the value of biomass was observed as the time went by eventhough the glucose value rose to around 20 mg/ml that did not effect biomass concentration nor ethanol production. However, when the yeast extract pulse was carried out, the biomass value increased sigmoidally with time, reaching values around 1 g/l. The increase in biomass affected positively the production of ethanol as it followed the trend of biomass although to a less extent as its values changed from 16 up to 22 g/l. Yet an efficiency ethanol conversion value of 90% was reached.

As far as ethanol production by *Zymomonas mobilis* CP4 under nitrogen-limited and glucose 5%(w/v) is concerned it can be said that such condition is inefficient as satisfactory values of ethanol were only achieved when the nitrogen restriction was temporarily removed. Thus the further experimentation with the chemostat utilised glucose at 5%(w/v) but the nitrogen limitation was removed by increasing the amount of the yeast extract to 1%(w/v).

### 3.2.3 CHEMOSTAT GROWTH UNDER GLUCOSE LIMITATION AT 5% (w/v)

Chemostat culture of *Zymomonas mobilis* CP4 was carried out under carbon limited conditions at 5%(w/v) glucose with yeast extract at 1%(w/v) in the medium.

Steady states were obtained over different dilution rate values as shown in figure 3.2.3 and table 3.2.3

Maximal values of biomass and ethanol were respectively 1.09 and 21.1 g/l at dilution rate 0.22 $h^{-1}$. Biomass and ethanol yield were 0.022 and 0.43 g/g respectively. The latter represents a value of conversion to ethanol efficiency of 85%. The maximum volumetric productivity of 5 g/l/h was observed at dilution rate 0.27 $h^{-1}$ but the value of conversion to ethanol efficiency started falling at that same value of dilution rate.

These results can be compared with those of Lee et al. (47) who grew *Zymomonas mobilis* ATCC 31821 with 6% (w/v) glucose medium at temperature 30° C. In their studies, at dilution rates up to 0.4 $h^{-1}$, glucose limitation occurred and ethanol values were around 30 g/l and biomass values were also superior (around 2 g/l) which led to values of biomass yield higher than 0.03 g/g.

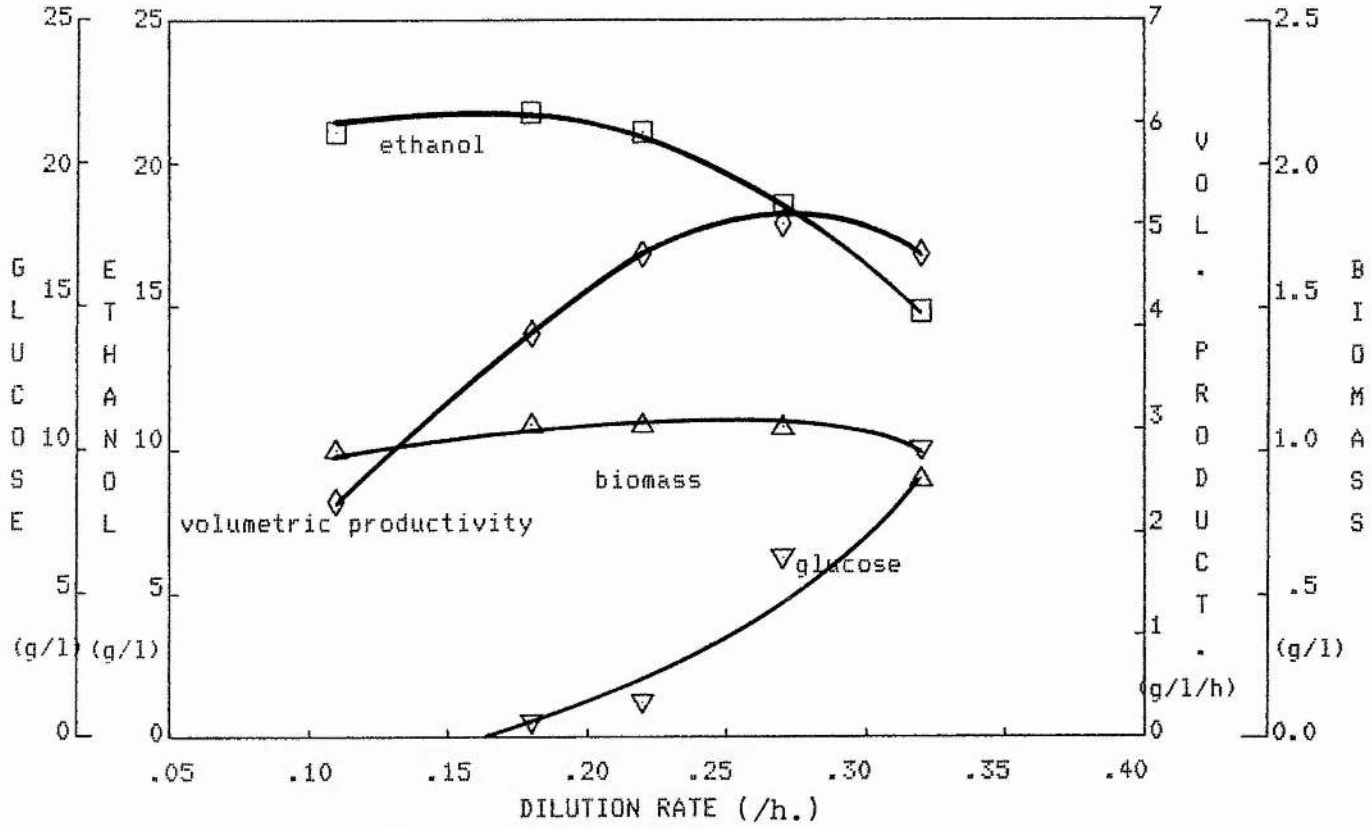



Fig. 3.2.3 Steady state data of continuous culture of Z. mobilis CP4. The culture was under glucose limitation of 5%(w/v) and 1%(w/v) yeast extract medium at temperature of 35 C.

Table 3.2.3 Continuous fermentation of glucose (51, 3 g/l ) by Z.mobilis CP4 at different dilution rates.

| D | Sr | X | [P] | V.P. | $Y_{x/s}$ | $Y_{p/s}$ | Conversion Efficiency |
|---|---|---|---|---|---|---|---|
| ($h^{-1}$) | (g/l) | (g/l) | (g/l) | (g/l/h) | (g/g) | (g/g) | % |
| 0.11 | n.d. | 1 | 21.1 | 2.3 | 0.019 | 0.41 | 82 |
| 0.18 | 1 | 1.09 | 21.8 | 3.93 | 0.022 | 0.43 | 85 |
| 0.22 | 2.4 | 1.09 | 21.1 | 4.7 | 0.022 | 0.43 | 82 |
| 0.27 | 12.5 | 1.08 | 18.5 | 5 | 0.028 | 0.48 | 72 |
| 0.32 | 20 | 0.9 | 14.8 | 4.7 | 0.029 | 0.47 | 58 |

n.d. : not detectable

### 3.2.4 TRANSIENT OPERATION TECHNIQUE

Microbial production in practical applications strongly depends on the extracellular parameters of the system which are of the physical and chemical nature.

Whereas the chemical parameters are represented by the nutrients and other components, the physical ones depend on the features of the equipment such as cultivation method and type of equipment selected.

So far in this work, the chemical parameters approach dealt with the usage of different sources of carbon for ethanol production whereas the equipment used was batchwise and chemostat. Glucose was selected due to the best results achieved. However when its concentration was elevated, values of productivity dropped.

It was decided for further experimentations a modification in the cultivation method. The modification of continuous cultivation chosen was transient reactor technique which was carried out by subjecting the microorganism to defined transient conditions of growth. This was implemented that such approach could lead to improved ethanol productivities as it has been known for over 40 years that periodic operation of a chemical system is sometimes superior to steady-state operation (71). Thus, by applying continuous changes in a particular system parameter, it is feseable that industrial bioreactors could

be made to increase product yields, an idea that has recently been proposed based on laboratory findings (72).

In industry there are few examples of fermentation that run both continuously and under what coulb be described as purposely transient condition (e.g., fed-batch-type fermentations). Examples of biological reactors operating continuously under transient conditions are those used for the production of penicillin (7) and cephalosporin (103) in repeated fed-batch culture. In these cases, both the period of antibiotic production and the overall culture productivity have been demonstrated to be increased by this type of operation.

Transient reactor operation (71) includes all periods in which environmental conditions change other than lag-phases. In analogy to chemical reactor operation (7) four broad classes of periodic operation were defined as shown in figure 3.2.4

First, a process life cycle considers the long-term operation of a reactor where a cycle may be determined by poisoning of a catalyst. In all instances of biological reactor operation this type of cycle will be short except, perhaps, where immobilized enzymes systems are involved.

The other classes consider the perturbed process in more detail.

Relaxed steady-state operation occurs when a system is unable to follow rapid cycling and enters what is termed a

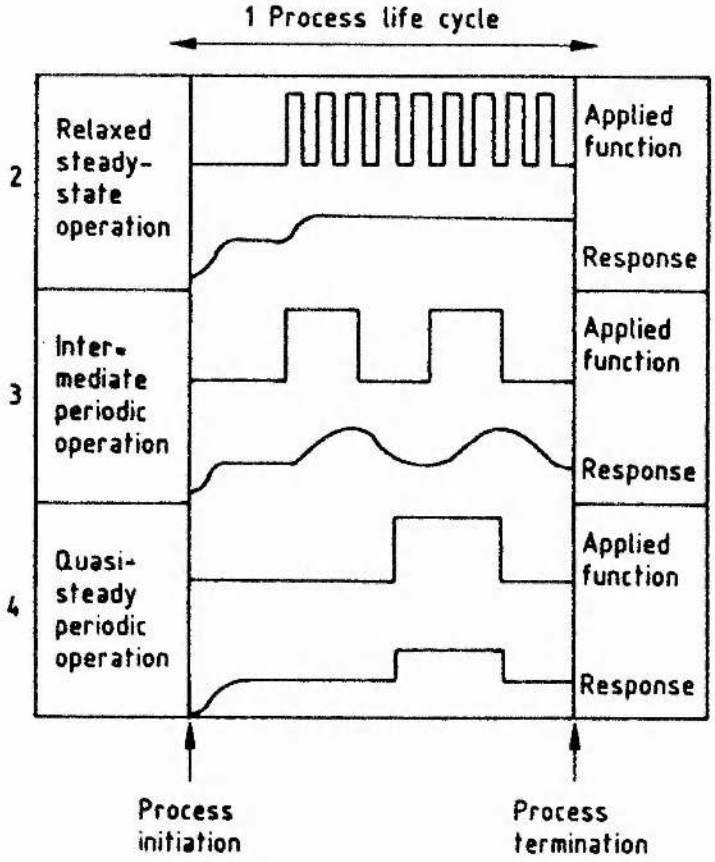



Fig. 3.2.4 The four classes of transient (periodic) reactor operation as defined by Bailey(7). Typical reactor output responses are shown for the four classes.

"relaxed" steady-state, a system towards a time invariant value.

Quasi-steady periodic operation is the opposite to relaxed steady-state operation and considers a process under time-invariant conditions, i.e., when the period of the input oscillation is large compared with the system response time; the system yields a steady-state relationship with the input at any time. Here, the mechanisms is fully adapted and it behaves as if the organisms were at steady-state with the particular environmental conditions. This is true for "balanced" growth in continuous cultures applications, the dynamics of a given mechanism are not important.

Intermediate periodic operation lies between both extremes and covers systems where the response time is of the same order as the imposed function cycle time so that "resonance" can happen. Here, the state of the mechanism changes dynamically and does not relate directly to the environment conditions at the moment considered.

Transient reactor operation have recently been summarised in review articles presented by Pickett (72), Barford et al. (10), Pickett et al.(73), Daizzer and Grady (25), Sherrard and Lawrence (87) and Chi and Howell (22).

3.2.4.1 TRANSIENT OPERATION CHEMOSTAT UTILISING GLUCOSE AT 2 AND 5%, AND 2 AND 10%, AND 2 AND 20% (W/V) FOR AMPLITUDE VALUES AT DIFFERENT CYCLE TIMES.

In this first set of experiments, the culture of *Zymomonas mobilis* CP4 was grown under continuous transient chemostat which was operated with alternating glucose amplitudes of 2 and 5%, 2 and 10%, and 2 and 20% (w/v), and cycle time values of 2, 4 and 6 hours. The ethanol production was compared to data obtained in simple chemostat operation grown with 3.5%, 6% and 11% (w/v) glucose, respectively. The value of dilution rate was fixed as 0.2 $h^{-1}$ for both techniques. Yeast extract was used at 1% (w/v) and all other components of the medium kept at their original concentrations as previously described.

The results obtained under transient operation are shown in figures 3.2.4.1.(a), 3.2.4.1.(b) and 3.2.4.1.(c). Both transient operation and simple chemostat chemostat techniques are compared in figure 3.2.4.1.(d).

Little has been published on the response of a microbial culture subjected to a well-defined and repeated transient conditions. Sundstrom et al. (95) tested the effect of varying the substrate concentration sinusoidally in an activated sludge reactor with biomass feed-back, but the results obtained by these workers were difficult to interpret in physiological terms since mixed, mainly unindentified cultures were used growing on an undefined medium.

Borzani et al.(16) using an essentially undefined substrate of sugar cane molasses, examined the response of the yeast *Saccharomyces cereviasiae* to simple and complex cyclic variations in substrate concentration. When they used a square-wave perturbation, the dry weight of their culture varied sinusoidally in a manner qualitatively similar to the fluctuations in turbidity that it was observed in the present work.

A general observation from the figures 3.2.4.1.(a), (b) and (c) is that as the frequency of glucose cycling was increased (i.e. the cycle decreased), the magnitude of the observed oscillations in biomass and ethanol decreased until it became difficult to detect any change. When the culture was under cycle time of 2 hours, the values of biomass and ethanol were kept constant. It did not matter the magnitude of the difference between the two glucose concentrations used was big (2 and 20%) or small (2 and 5%) and no apparent effect could be observed. The culture behaviour tended more toward a steady state condition. In other words, the culture showed features of being under relaxed steady state operation (according to figure 3.2.4 classification).

When cycle time value of 2 hours was used, the cells were apparently unable to respond to rapid changes in the growth environment, the changes occuring too rapidly for their metabolism to adjust accordingly inbetween. Alternatively, the cells could be responding to the imposed conditions but their response was too small to be detected experimentally.

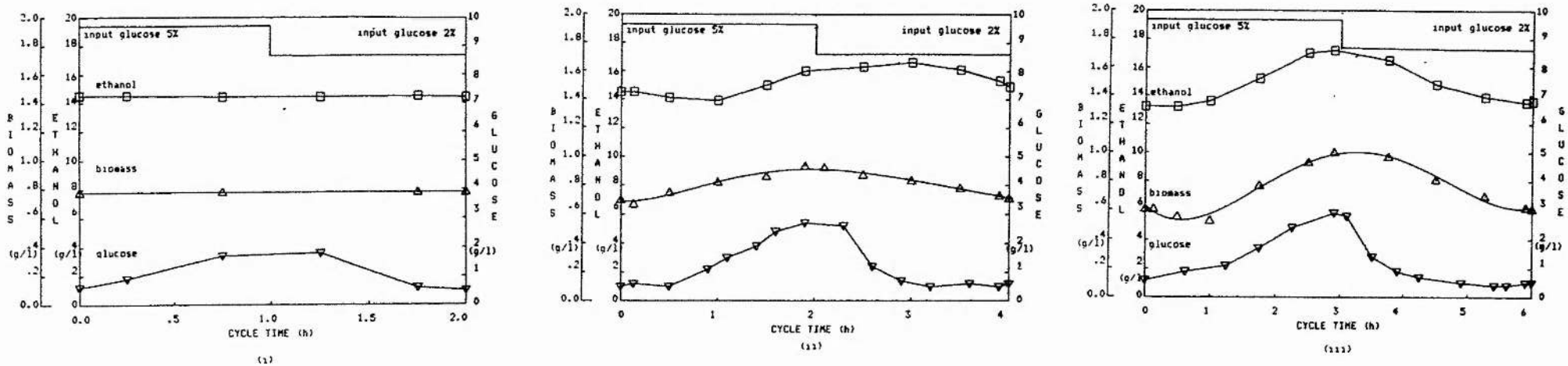



FIGURE 3.2.4.1(a) Kinetics parameters for Z. mobilis CP4 growing in chemostat culture and subjected to variations of fed glucose of 2 and 5% (w/v) and values of cycle time of 2h (i), 4h (ii) and 6 hours (iii). Dilution rate 0.2 h-1

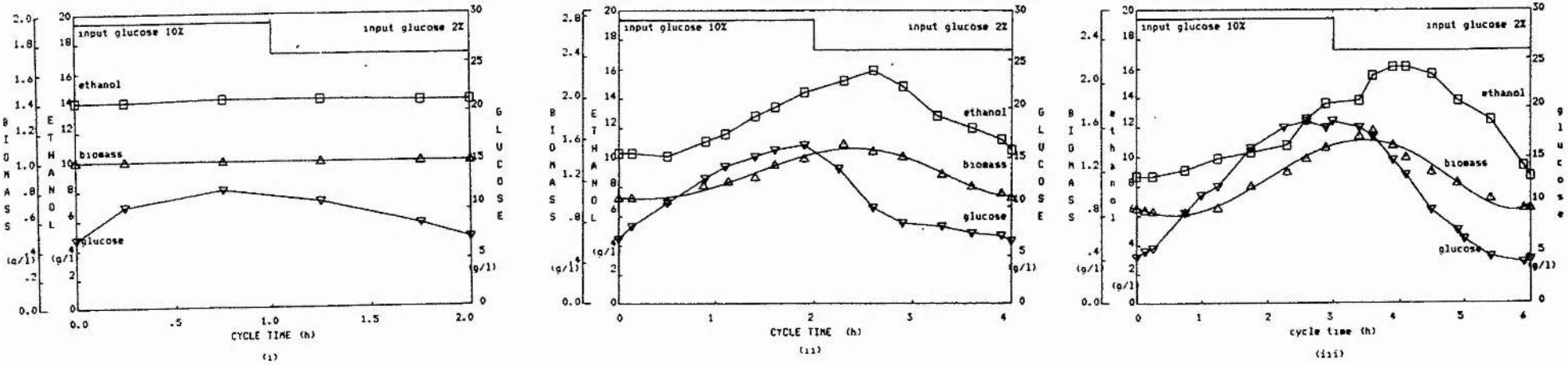



FIGURE 3.2.4.1(b) Kinetics parameters for Z. mobilis CP4 growing in chemostat culture and subjected to variations of fed glucose of 2% and 10%(w/v) and values of cycle time of 2h (i), 4h (ii) and 6 hours (iii). Dilution rate 0.2 h-1

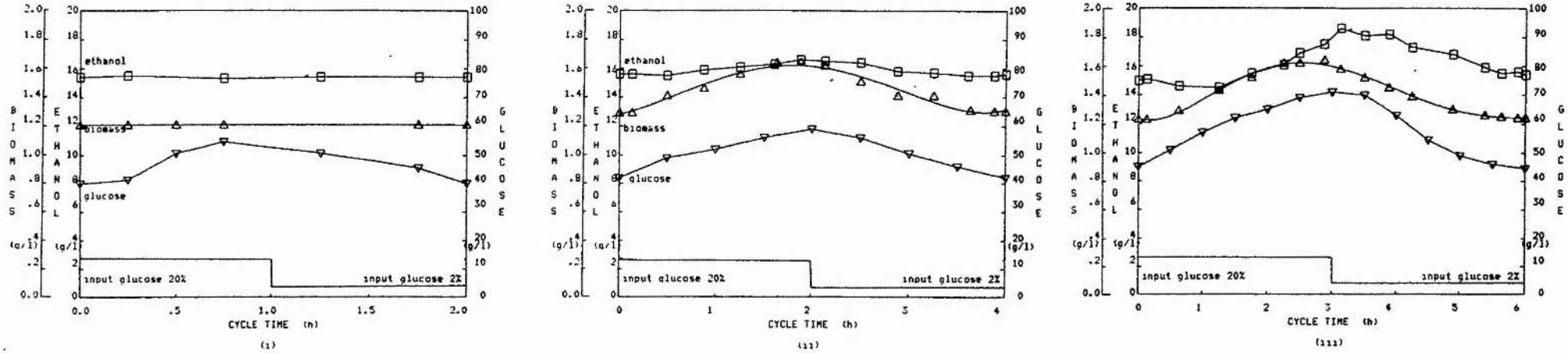



FIGURE 3.2.4.1(c) Kinetics parameters for Z. mobilis CP4 growing in chemostat culture and subjected to variations of fed glucose of 2 and 20% (w/v) and values of cycle time of 2h (i), 4h (ii) and 6 hours (iii). Dilution rate 0.2 h-1

The values of biomass for cycle time of 2 hours were 0.78, 1 and 1.21 g/l when the chemostat operated with alternating glucose amplitude of 2 and 5%, 2 and 10% and 2 and 20%(w/v), respectively.

Another general comment is that the glucose in the reactor had its value increased as the system was being fed with the higher glucose medium while its value decreased when the lower glucose medium was used. This is valid for any cycle time or amplitude carried out.

The appliance of cycle time of 4 hours produced smooth responses from the organisms. Smooth oscillations on values of biomass and ethanol could be seen. The values of biomass oscillated from 0.7 to 0.93 (alternating glucose amplitude of 2 and 5%), from 1.01 to 1.56 (alternating glucose amplitude of 2 and 10%) and from 1.3 to 1.65 g/l when alternating glucose amplitude of 2 and 20% were used.

When cycle time of 6 hours was used, the behaviour of the culture was clearer when compared with cycle time of 4 hours as the oscillations on values of biomass and ethanol were more pronounced whatever the alternating glucose concentrations were applied. The system was able to respond and changed dynamically according to the input of different concentrations of glucose.

The oscillatory response of the culture, with minima values of biomass 0.61; 0.83; and 1.23 g/l (for alternating glucose amplitude of 2 and 5%, 2 and 10%, and 2 and 20%, respectively) occurred just after the change to higher input nutrient concentration. At that point the culture produced a

lag-phase like stage followed by a sigmoidally increase with time reaching its highest values. The maxima values of biomass were 1 for 2 and 5% glucose amplitude, 1.57 for 2 and 10% glucose amplitude and 1.64 g/l for 2 and 20% glucose amplitude.

Over the second half of the cycle time, after the change to low input nutrient concentration, the values of biomass dropped. The response to shift-down in glucose concentration was always faster than the response to shift-up.

As far as conversion efficiency to ethanol is concerned the same values of glucose amplitude used can be gathered despite of the different cycle time which were carried out. So when the alternating glucose concentrtion of 2 and 5% were used, the similar values of 84, 85 and 89 of conversion efficiency values (corresponding to cycle time of 2, 4 and 6 hours) were found.

When the alternating glucose concentration of 2 and 10% were used, the similar values of 52, 47 and 47% of conversion efficiency (corresponding to cycle time of 2, 4 and 6 hours) were found. The comparison here with simple chemostat running with 6% (w/v) glucose medium as shown in figure 3.2.4.1.(d) shows that both techniques produce similar results.

When glucose at 2 and 20% were used, the similar values of 28, 30 and 30% of conversion efficiency values (corresponding to cycle time of 2, 4 and 6 hours) were found. The comparison here with simple chemostat fed with 11%(w/v) glucose medium shows that an inverted situation as

simple chemostat reached higher values of 42% as shown in figure 3.2.4.1.(d).

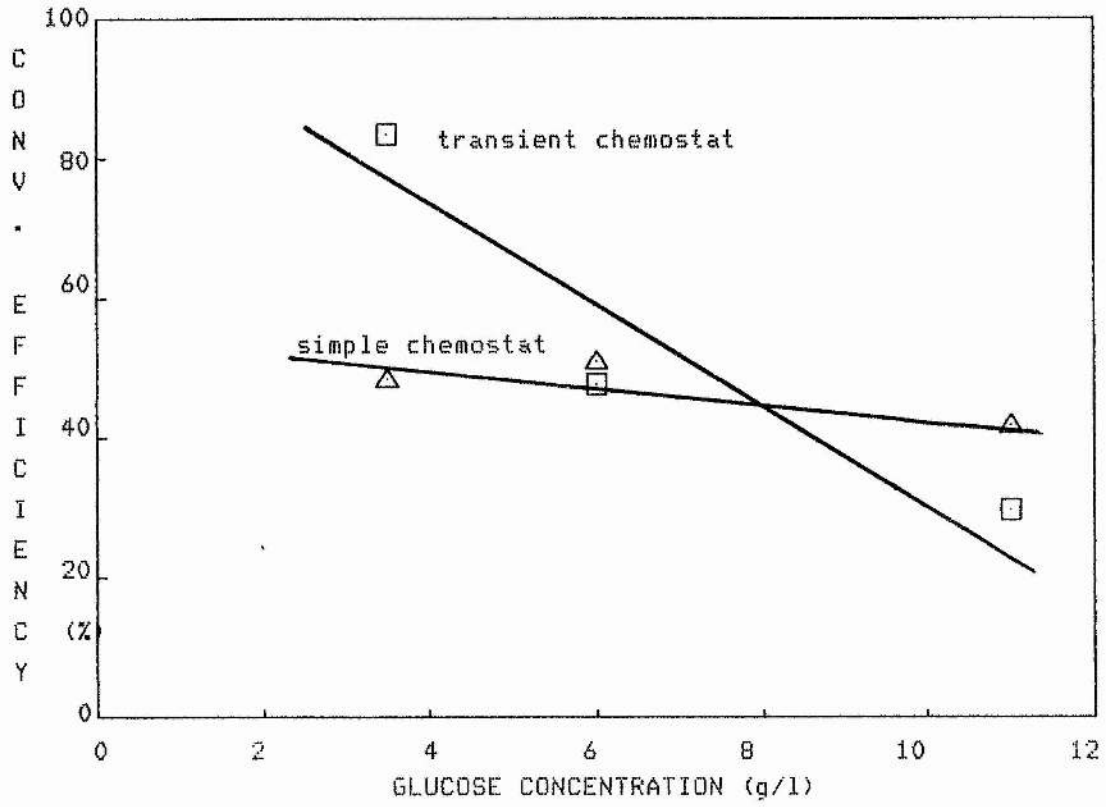



Fig. 3.2.4.1(d) Comparison of conversion Efficiency of glucose to ethanol under transient chemostat operation using alternating glucose concentration of 2 and 5, 2 and 10 and 2 and 20% (Only mean values of different cycle time are used) and simple chemostat with glucose at 3.5; 6 and 11% (w/v) medium.

In the further set of experiments it was decided to test the transient operation where the concentration of glucose was in a more realistic one, nearer of what is used in alcohol factory. Two higher pairs of glucose concentrations of 8 and 11% (w/v) and 8 and 16% (w/v) were used in the fermentation carried out.

3.2.4.2 TRANSIENT OPERATION CHEMOSTAT OF *Z. MOBILIS* CP4 UTILISING 8 AND 11% AND 8 AND 16% GLUCOSE MEDIUM AT CYCLE TIME VALUE OF 2 HOURS.

In this set of experiments, *Zymomonas mobilis* CP4 was grown under continuous transient chemostat which was operated with alternating glucose amplitude of 8 and 11% and 8 and 16% (w/v). For comparison simple chemostat running with glucose 9.5 and 12% (w/v) medium were carried out . As the value of cycle time played an unimportant rule in the first set of experiment described in sesseion 3.2.4.1, its value was fixed as 2 hours for the present fermentations.

To prevent nitrogen limitation, the yeast extract concentration was increased to 2% in the medium while other components of it kept at their original concentration as previously described.

The figure 3.2.4.2 shows steady state conditions for the two pairs of alternating glucose used under continuous transient conditions and table 3.2.4.2 shows the values of conversion efficiency to ethanol for both continuous transient and simple chemostat operation used.

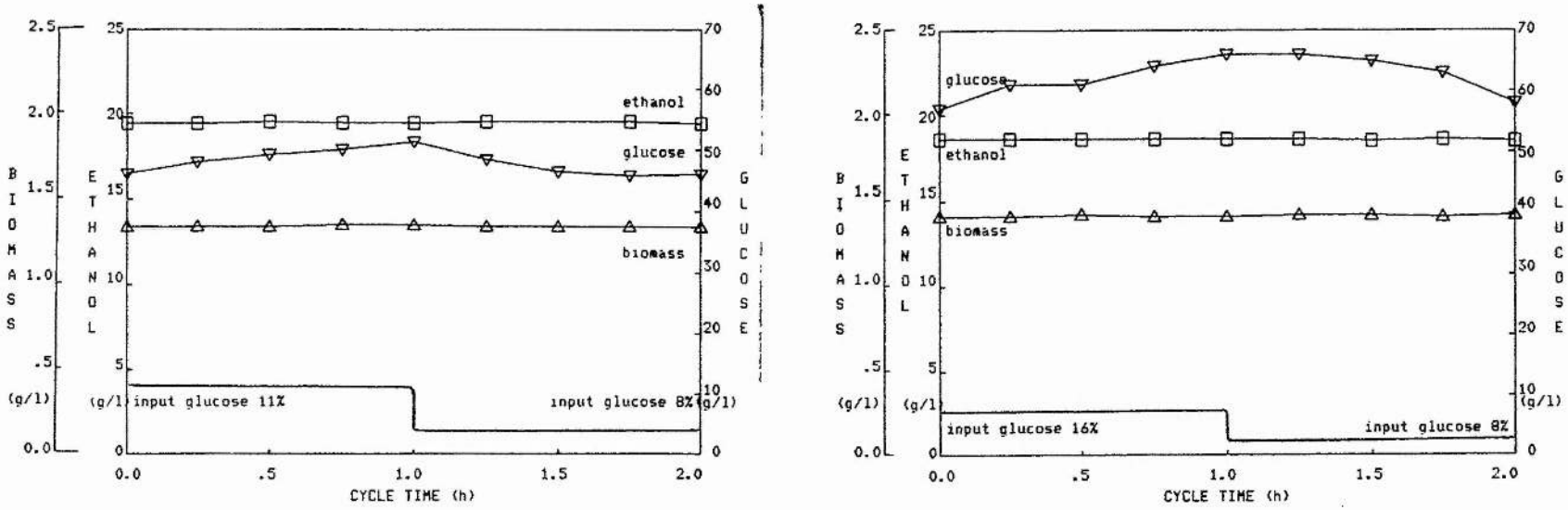



Fig. 3.2.4.2 Kinetic parameters for Z. mobilis CP4 growing in chemostat culture and subjected to variations of fed glucose 8% and 11% (left) and 8% and 16% (right) at fixed cycle time value of 2 hours.

Biomass reached similar values of 1.3 and and 1.4 g/l for alternating concentrations of glucose values of 8 and 11% (w/v) and 8 and 16% (w/v) respectively while values of ethanol showed to be also similar as they were around 20 g/l for both pairs of glucose concentrations used.

The non-glucose utilised present in the fermenter reached the high values of 50 and 66g/l for glucose amplitude values of 8 and 11% and 8 and 16% respectively which shows that glucose was poorly utilised by the bacteria and largely washed out the system.

When both continuous transient and simple chemostat are compared as table 3.2.4.2 does, it can be said that neither technique employed gave satisfactory results. Both techniques were quite unefficient as the rate of glucose conversion to ethanol were as low as 40% and 29% respectively for glucose amplitude values of 8 and 11% and 8 and 16% when transient operation was used and 48% and 45% for 9.5% and 12% (w/v) glucose media used under simple chemostat operation.

As the conversion efficiency values were not satisfatory it was decided to check the system in respect of rate-limiting nutrients by carrying out pulsing of single components of the medium in the chemostat. Magnesium, Potassium, Ammonium and yeast extract were the compounds to be tested . In order to check the effect of ethanol on the culture, a pulse utilising ethanol was also carried out.

Table 3.2.4.2 Comparison of conversion efficiency of glucose to ethanol under transient chemostat operation (at fixed cycle time value of 2 hours) and single chemostat technique by Z.mobilis CP4. Dilution rate: 0.2h-1

| transient operation | | simple operation | |
|---|---|---|---|
| glucose amplitude (g\l) | conversion efficiency (%) | conversion efficiency (%) | glucose concentration (g\l) |
| 8 and 11 | 40 | 48 | 9.5 |
| 8 and 16 | 29 | 45 | 12 |

### 3.2.5 DELTA-TYPE OF PULSE UTILISING MINERAL SALTS, YEAST EXTRACT AND ETHANOL.

Chemostat culture of *Zymomonas mobilis* CP4 was grown under 9.5% glucose and 2% yeast extract medium at fixed dilution rate value of 0.2 $h^{-1}$. When steady state was achieved, a series of pulsing utilising 10 ml of each the following compounds: 1.5g $MgSO_4$, 1.5g $KH_2PO_4$, 1.5g $(NH_4)_2SO_4$, 3 g Yeast extract and 100% ethanol, were carried out.

#### 3.2.5.1 MAGNESIUM DELTA-TYPE PULSE.

The figure 3.2.5.1 shows the magnesium pulse carried out on the culture.

Medium composition plays an essential role of a successful laboratory experiment, pilot-scale development and manufacturing process. The constituents of a medium must satisfy the elemental requirements for cell biomass and metabolite production and there must be an adequate supply of energy for biosynthesis and cell maintenance.

Magnesium is required in the medium composition for playing a very important role in the action of many enzymes, particularly those involved in glucose metabolism and many ATP-dependent reactions. However high concentrations of magnesium in a ethanol producing process utilising molasses leads to low productivity and Skotnicki et al. (88) have reported that high magnesium concentration in the medium inhibits the growth of *Zymomonas mobilis* ZM4 while Cromie and Doelle (23) have

published that increasing the amount of magnesium above 6g/l did not improve glucose utilisation.

When the pulse utilising magnesium was carried out it was demonstrated that the culture was neither under magnesium limitation nor being inhibited by it as the figure 3.2.5.1 shows, the culture was not disturbed by the sudden rise of magnesium.

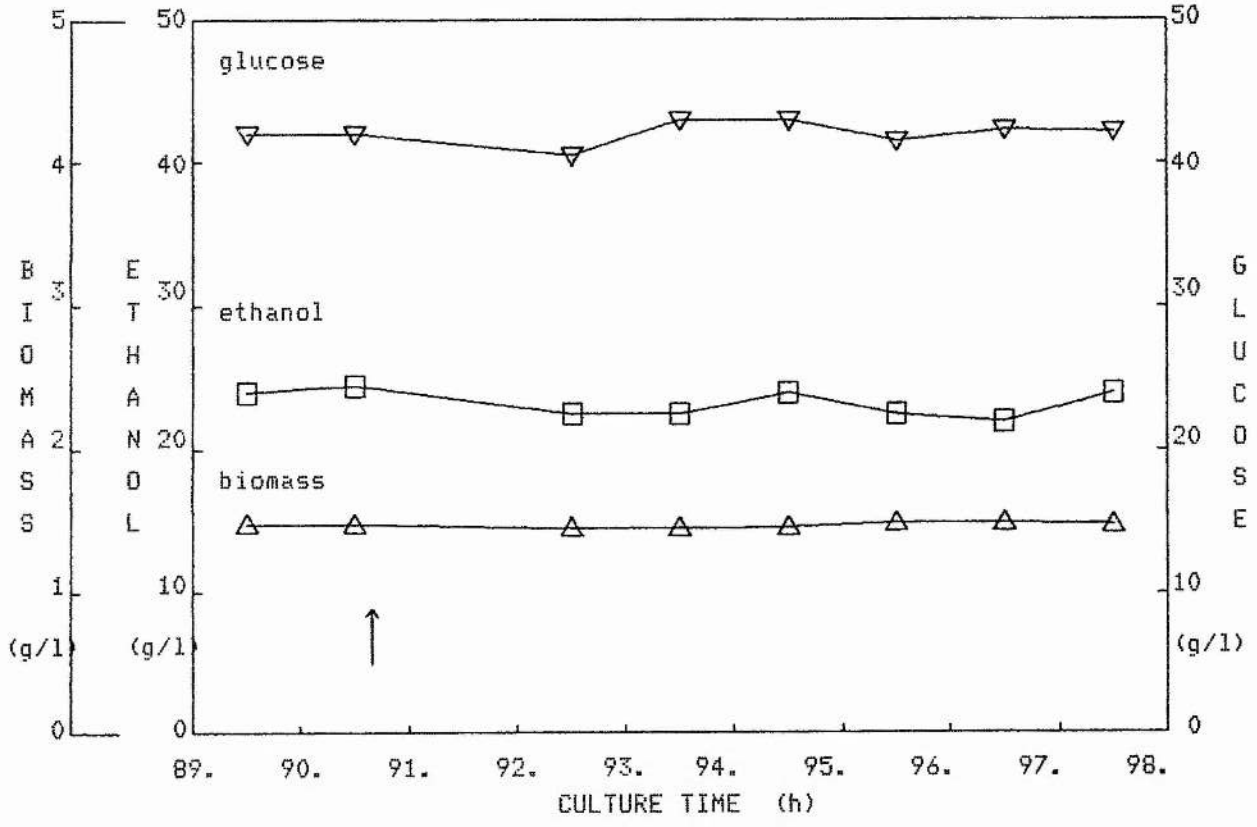



Fig. 3.2.5.1 Magnesium pulsing in chemostat culture of Z. mobilis CP4 grown in 9.5%(w/v) glucose and 2%(w/v) yeast extract medium at 35 C and D= 0.2 /h. The arrow indicates when the pulse was carried out.

3.2.5.2 POTASSIUM DELTA-TYPE PULSE.

The figure 3.2.5.2 shows the potassium pulse carried out on the culture.

Potassium is listed amongst those inorganic ions which are required in substantial quantity. Much of the potassium seems to be bound up with the RNAs so that the potassium requirement is increased by factors such as growth rate which increases the RNA content of the biomass. Potassium ions function as coenzymes and probably act as cations in the structure of RNA and other anionic structures in the cell (75).

According to Skotnicki et al. (88) the growth and ethanol production by *Zymomonas mobilis* is poor due to the high concentration of this ion in molasses. Amorim and Campos (4) have mentioned that high concentration of potassium in molasses depresses by competition the uptake of other ions such as magnesium, zinc and manganese.

When the pulse utilising potassium was carried out it was shown that the culture was neither under potassium limitation nor being inhibited by it as the figure 3.2.5.2 shows, the culture was not disturbed by the sudden rise of potassium.

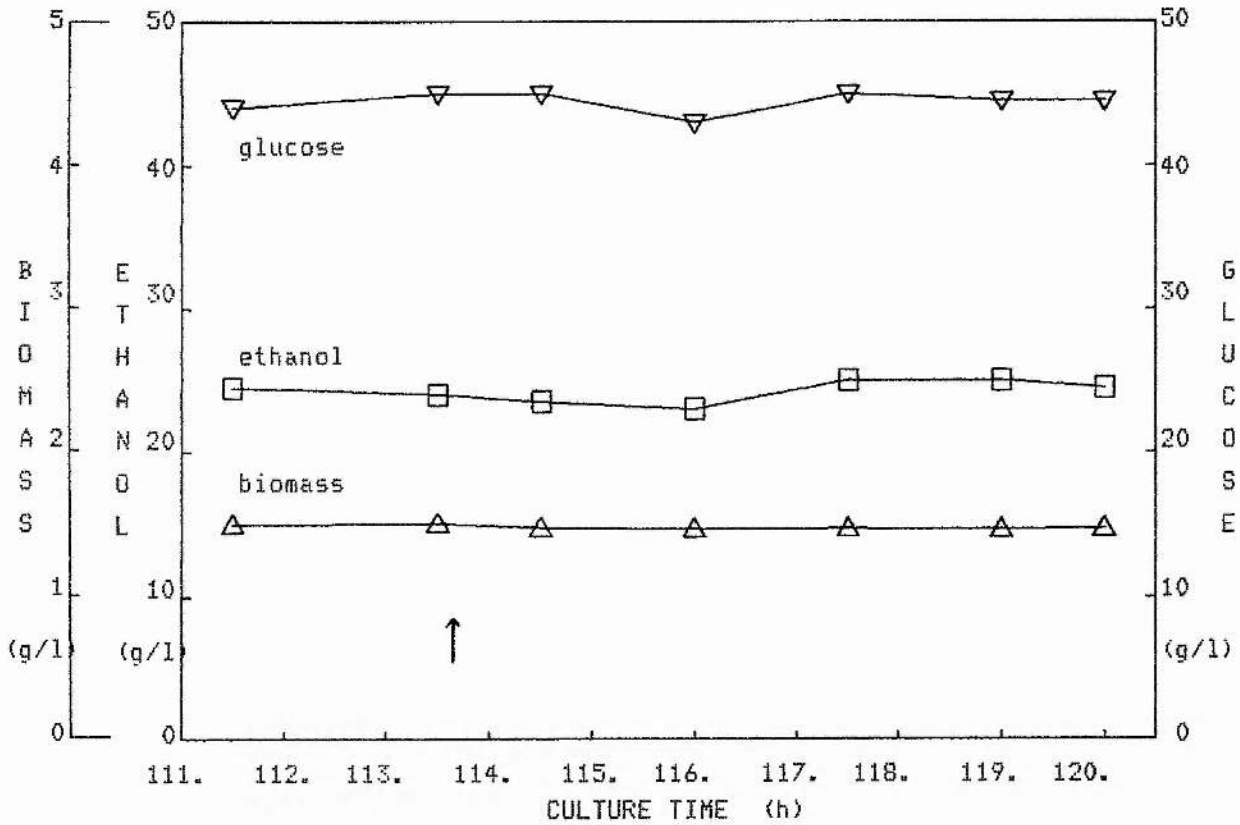



Fig. 3.2.5.2 Potassium pulsing in chemostat culture of Z. mobilis CP4 grown in 9.5%(w/v) glucose and 2%(w/v) yeast extract medium at 35 C at 35 C and D= 0.2/h. The arrow indicates when the pulse was carried out.

### 3.2.5.3 AMMONIUM DELTA-TYPE PULSE

The figure 3.2.5.3 shows the ammonium pulse carried out on the culture.

As Alexandri et al.(2) have pointed out there is a total lack of knowledge about the uptake of aminoacids and other N-substrates in *Zymomonas mobilis*. They have published a study on aminoacid uptake by *Zymomonas mobilis* utilising glutamine as substrate while Galami et al.(34) have reported that ammonium sulphate could be used as a sole nitrogen source for growth of cells of *Z. mobilis* when a chemically defined minimal medium was utilised.

In this work, when the ammonium pulse was carried out there was no evidence that indicated the culture was under limitation or inhibition by ammonium sulphate.

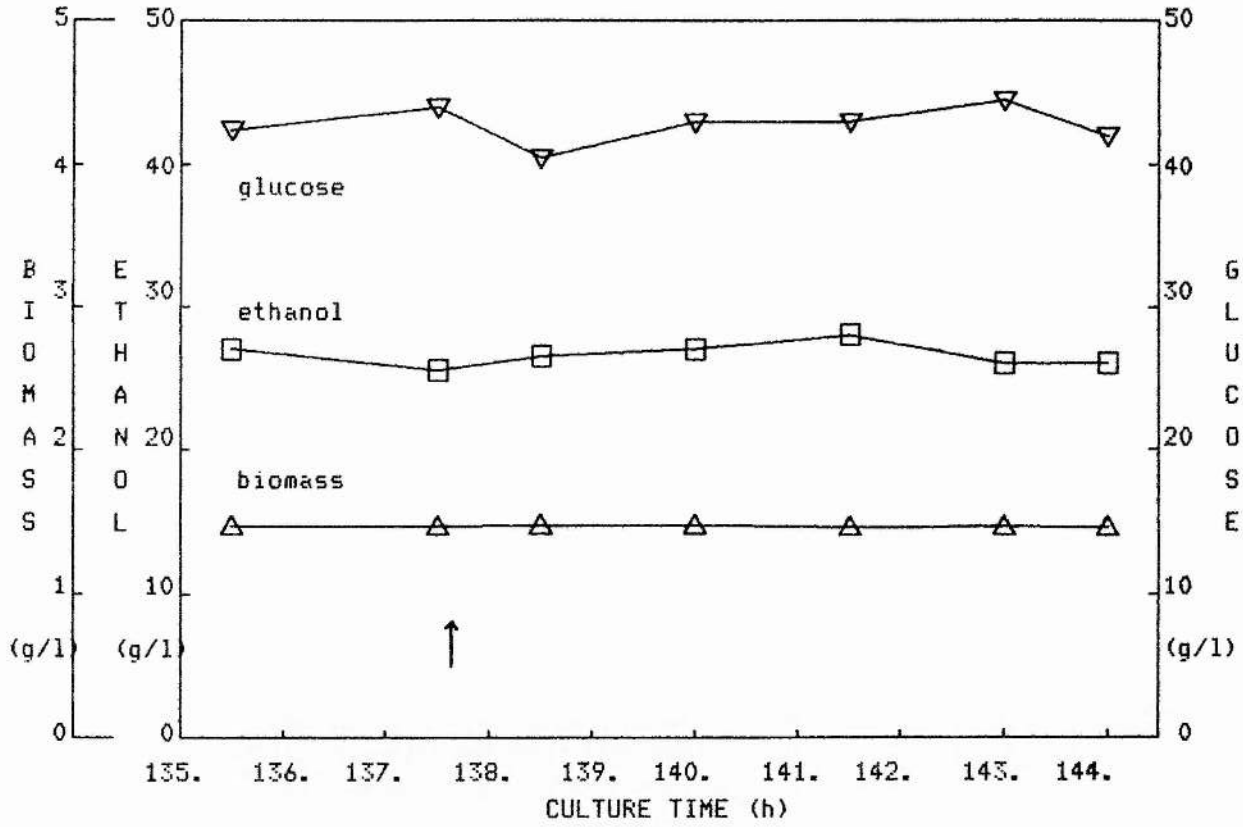



Fig. 3.2.5.3 Ammonium pulsing in chemostat culture of Z. mobilis CP4 grown in 9.5%(w/v) and 2%(w/v) yeast extract medium at 35 C and D=0.2 /h. The arrow indicates when the pulse was carried out.

### 3.2.5.4 YEAST EXTRACT DELTA-TYPE PULSE

The figure 3.2.5.4 shows the yeast extract pulse carried out on the culture.

Yeast extract can promote the growth of an array of microorganisms, both aerobic and anaerobic. It provides a variety of organic nitrogeneous constituents (partial breackdown products of proteins) which can fulfill the general nitrogen requeriments, and it also contains minerals and most of the organic growth factors likely to be required to microorganism. Yet when the yeast extract pulse took place there was no evident effect or disturbance upon the culture.

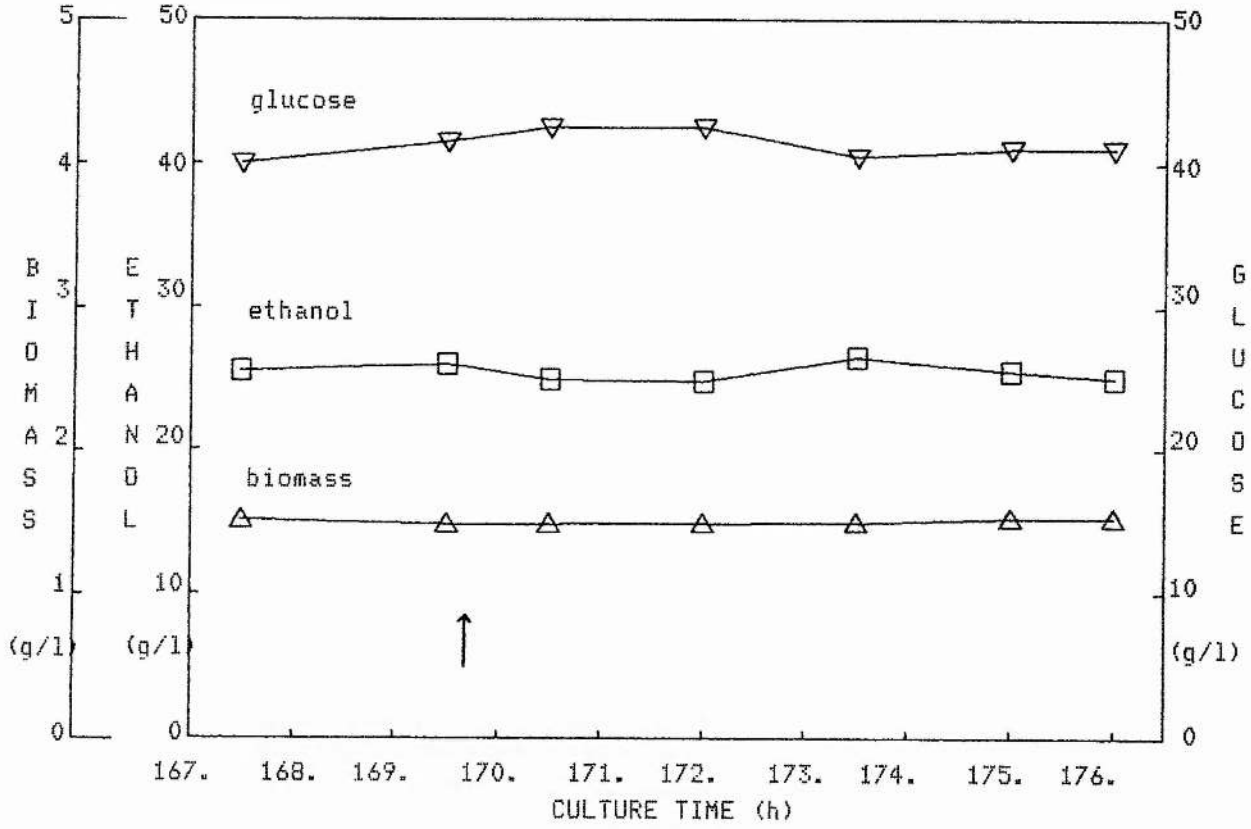



Fig. 3.2.5.4 Yeast extract pulsing in chemostat culture of Z. mobilis CP4 grown in 9.5%(w/v) glucose and 2%(w/v) yeast extract medium at 35 C and D=0.2 /h. The arrow indicates when the pulse was carried out.

### 3.2.5.5 ETHANOL DELTA-TYPE PULSE

The figure 3.2.5.5 shows the ethanol pulse carried out on the culture.

The accumulation of ethanol as an end product of fermentation is known to cause progressive inhibition in the rate of sugar conversion to ethanol by *Zymomonas mobilis* (20,44). This phenomenon is particularly important during commercial ethanol production and increases the time required to complete the conversion of sugar substrate to ethanol and limits the final concentration of ethanol achieved (42). Many studies have hypothesized that the inhibition is due to direct action of ethanol on key enzymes of glycolysis and ethanol production involving feedback inhibition or enzyme inactivation (14,41,44,63,69). Osman and Ingram (70) have proposed that inhibition of fermentation by ethanol in *Zymomonas mobilis* appears to result from increased leakage through the plasma membrane, allowing loss of cofactors and coenzymes. Three basic ways in which ethanol would be expected to decrease the effectiveness of the plasma membrane as a hydrophobic barrier: (i) by altering the coligative properties of the environment, (ii) by directly interacting with the membrane, and so decreasing the effectiness of the hydrophobic core of the membrane leading to increased membraqne permeability and membrane leakage, and (iii) by altering the dieletric properties of the environment.

When the ethanol pulse was carried out the negative effect on the culture could be observed as the biomass dropped and then later return to its previous value as ethanol was being washed out the system. This suggests that *Zymomonas mobilis* CP4 is particularly sensitive to high concentrations of ethanol present in the broth.

Cromie and Doelle (23) have emphasized the need to develop new designed cultivation techniques to suit the peculiarities of *Zymomonas mobilis* in order to increase ethanol production and substrate utilisation efficiency. Many publications have responded to that statement of Cromie and Doelle (23) and different fermentations techniques have been adapted and making possible the use of *Zymomonas mobilis* as promising ethanol-producer.

However, it seems that *Zymomonas mobilis* CP4 exhibits a considerable sensibility to ethanol as well as an incomplete metabolism of substrate when it is presented at high concentration. Tackling particularly the inhibitory effect of ethanol Bruce and Daugulis (19) and Bruce et al. (18) have successfully shown that extractive fermentation can be used to greatly improve the performance of *Zymomonas mobilis* in continuous culture without adversely affecting ethanol or biomass yields. The continuous removal of ethanol from the broth, by the cycling a solvent through the culture, the ethanol concentration was kept below the inhibitory effect and allowed the fermentation to continue at high reaction rate.

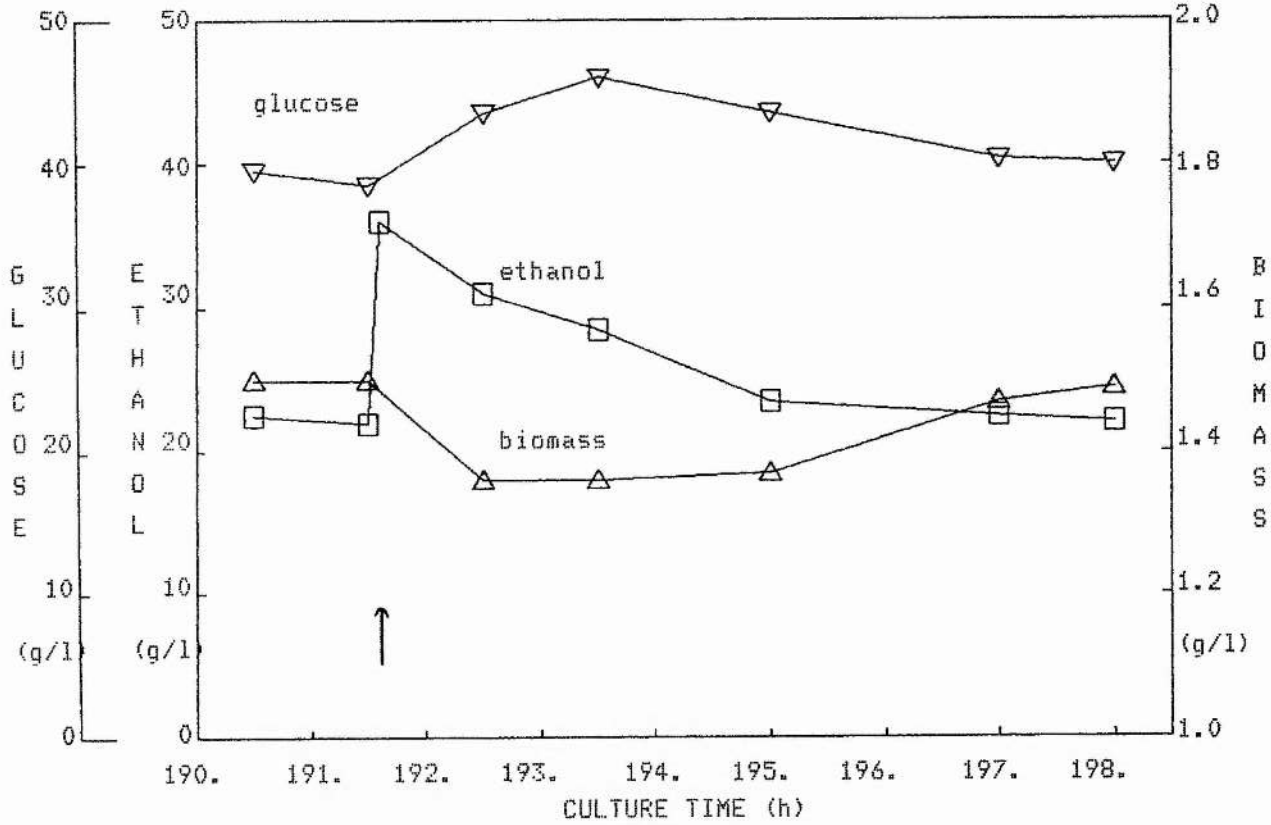



Fig. 3.2.5.5 Ethanol pulsing in chemostat culture of Z. mobilis CP4 grown in 9.5%(w/v) and 2%(w/v) yeast extract medium at 35 C and D= 0.2 /h. The arrow indicates when the pulse was carried out.

# 4. FINAL CONCLUSIONS

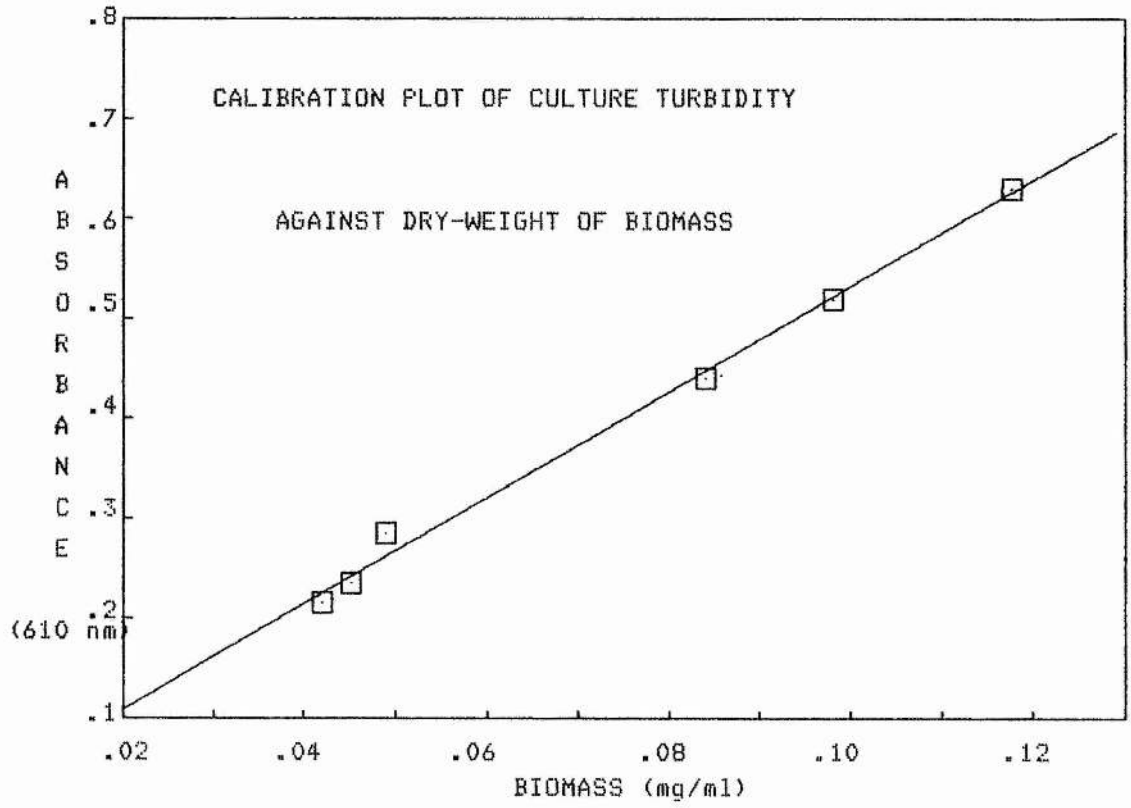
Ethanol production by *Zymomonas mobilis* CP4 under simple chemostat and continuous transient operation has been investigated.

Under carbon limitation condition with simple chemostatic operation, glucose, fructose and sucrose were tested at 2%(w/v) in the medium. At this concentration glucose was more efficiently utilised as conversion efficiency to ethanol value of 96% was achieved. However, glucose at 5%(w/v) under nitrogen limitation condition was not fully metabolised as 25% of feed glucose medium was wasted and the value of conversion efficiency to ethanol was around to 60%. When the nitrogen restriction was removed, values higher than 80% for conversion efficiency to ethanol were reached.

Under alternating glucose of 2 and 5%(w/v) medium, continuous transient operation achieved values of ethanol conversion higher than 80%. However, both continuous transient operation (utilising high alternating glucose concentration of 8 and 11% and 8 and 16%(w/v)) and simple chemostat operation running at 9.5% and 12%(w/v) glucose medium did not achieved satisfactory results as conversion efficiency values were as low as 40 and 29% for the former and 48 and 45% for the latter.

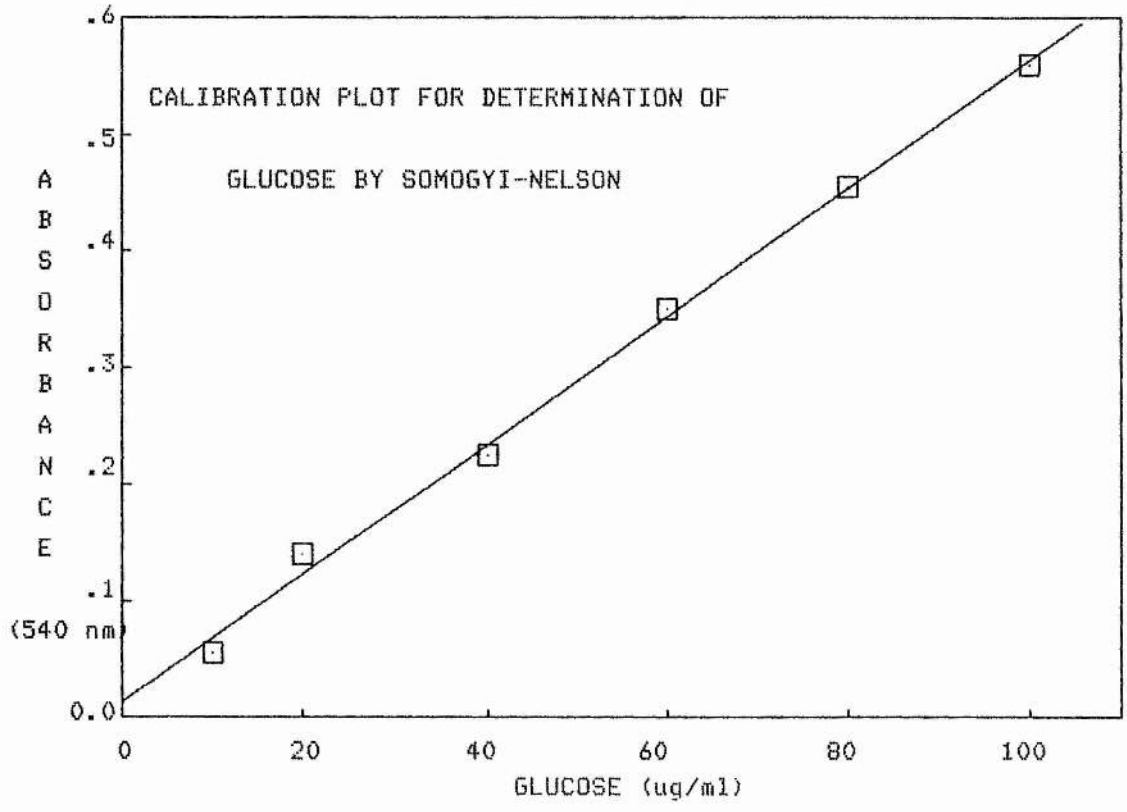
Pulsing the culture with mineral salts ($Mg^{++}$, $K^{+}$, $NH_4^{+}$) and ethanol, the culture showed no effect with mineral salts and a strong inhibitory effect on growth for ethanol.

APPENDIX 1



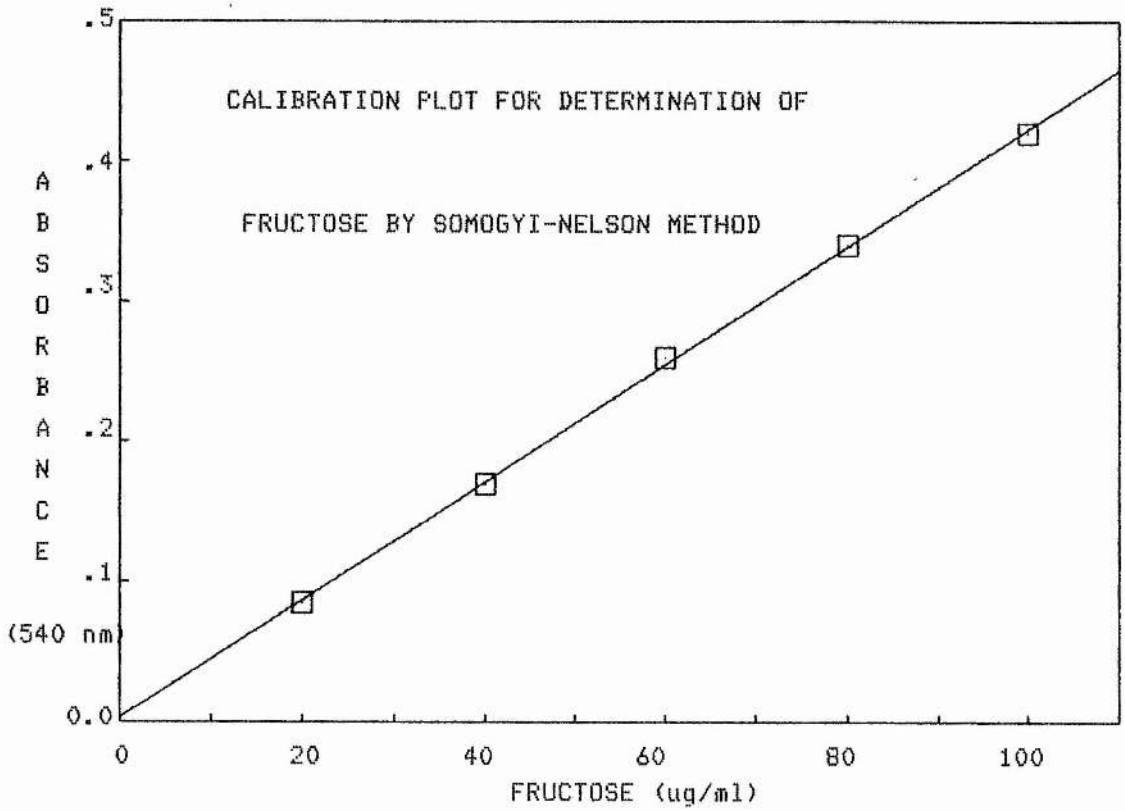

| | |
|---|---|
| Slope | 5.303041 |
| Y intercept | 2.340158E-03 |
| Correlation coefficient | 0.9973 |
| Standard error | 1.375842E-02 |

APPENDIX 2



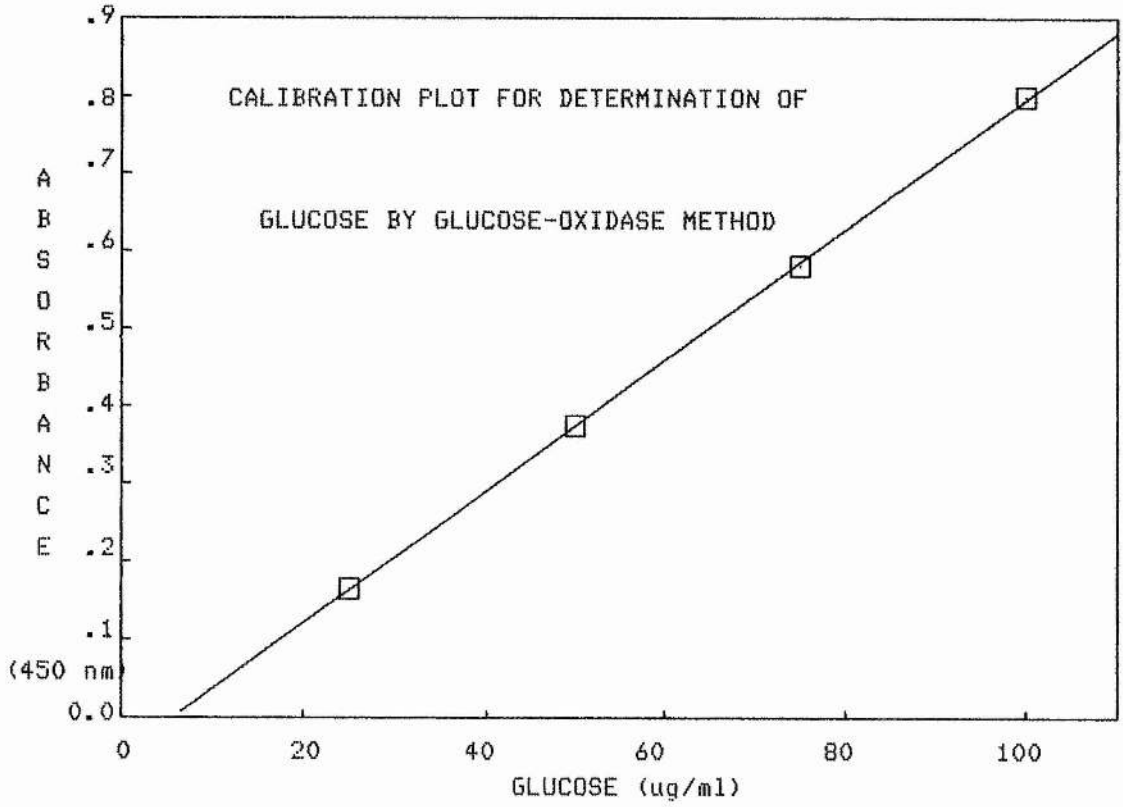

| | |
|---|---|
| Slope | 5.510958E-03 |
| Y intercept | 1.276716E-02 |
| Correlation coefficient | 0.9984 |
| Standard error | 1.206858E-02 |

APPENDIX 3



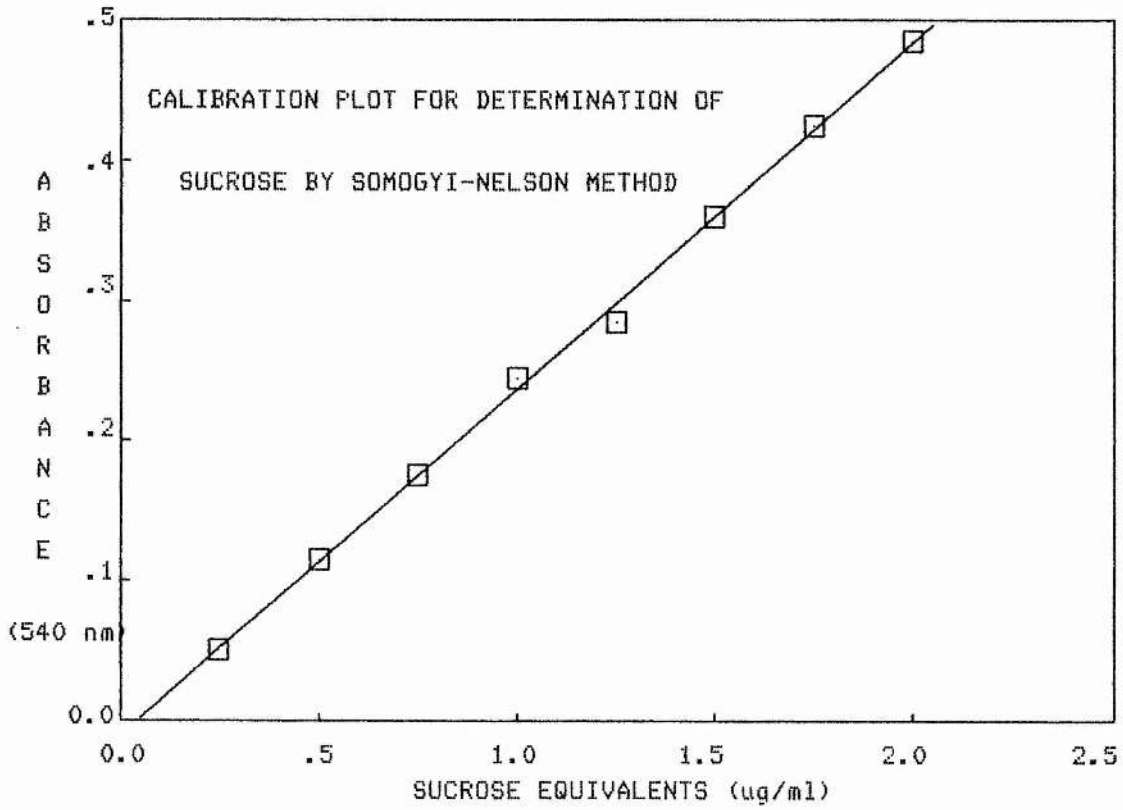

| | |
|---|---|
| Slope | 4.200001E-03 |
| Y intercept | 2.99995E-03 |
| Correlation coefficient | 0.9997 |
| Standard error | 3.651484E-03 |

APPENDIX 4



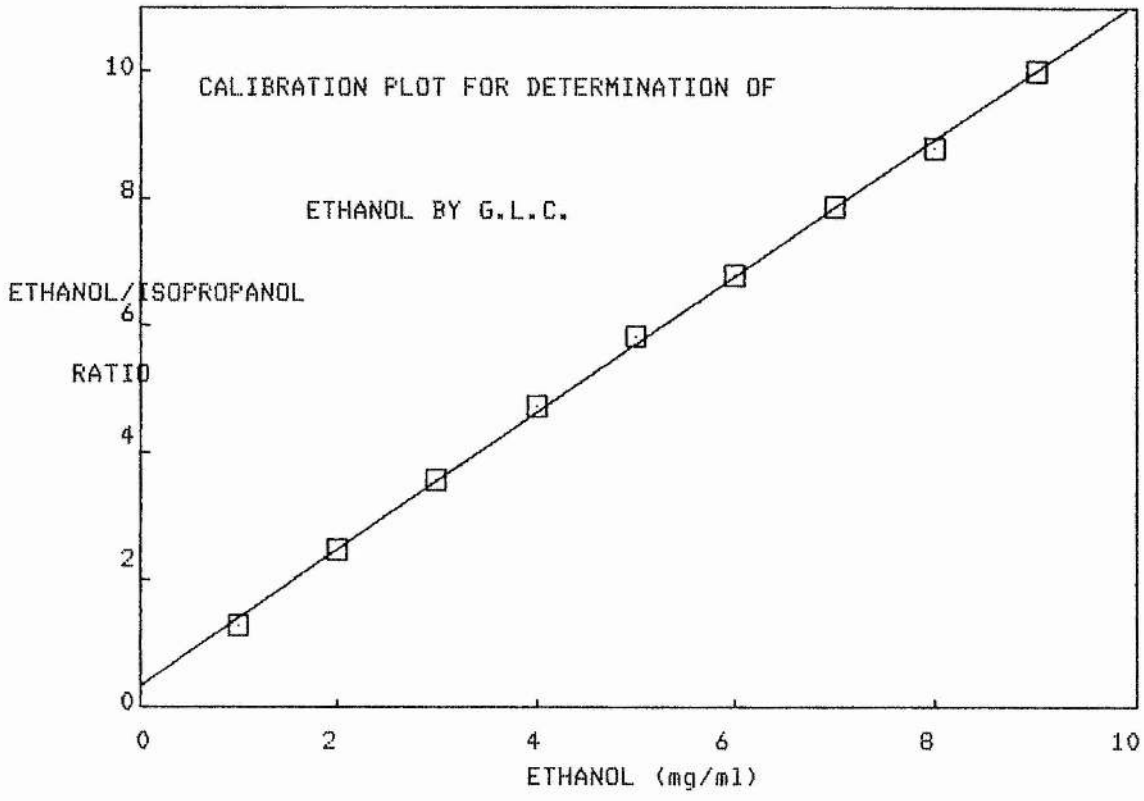

| | |
|---|---|
| Slope | 8.439997E-03 |
| Y intercept | -4.749984E-02 |
| Correlation coefficient | 0.9999 |
| Standard error | 4.743414E-03 |

APPENDIX 5




| | |
|---|---|
| Slope | .247143 |
| Y intercept | -1.053587E-02 |
| Correlation coefficient | 0.9992 |
| Standard error | 6.659232E-03 |

APPENDIX 6




| | |
|---|---|
| Slope | 1.076334 |
| Y intercept | .322776 |
| Correlation coefficient | 0.9996 |
| Standard error | 9.382016E-02 |

## 5.7 APPENDIX 7

Calculation of the maximum specific growth rate ($\mu_{max}$) of *ZYMOMONAS MOBILIS* CP4 grown in chemostat culture using the washout method.

In a chemostat, the biomass balance can be described as

$$dx/dt = \mu x - Dx = ( \mu - D )x$$

integrating

$$\int dx/x = ( \mu - D ) \int dt$$

$$\ln x - \ln x_0 = ( \mu - D )t \quad \ldots\ldots\ldots (1)$$

The Monod equation states that

$$\mu = \mu_{max} . S/(K_s + S)$$

Here $\mu_{max}$ is the maximum growth rate achievable when $S >> K_s$ and $K_s$ is that concentration of S at which the specific growth rate is half its maximum value.

When $S >> K_s$, then Monod equation becomes

$$\mu = \mu_{max}$$

and we can put $\mu = \mu_{max}$ in equation.....(1)

$$\ln x - \ln x_0 = ( \mu_{max} - D )t$$

we get

$$\ln X = ( \mu_{max} - D )t + \ln x_0$$

With D higher than $D_c$ the slope of the logarithmic plot is ( $\mu_{max}$ - D ); (where $\mu$ will be $\mu_{max}$)

Under carbon limitation with at 2%(w/v) and the dilution rate of the culture in steady state was 0.22 $h^{-1}$ (section 3.1.2). Then the dilution rate was shifted to 0.61 $h^{-1}$, a value well above of $\mu_{max}$ ,now

$$\text{slope} = (\mu_{max} - D)$$

$$-0.24 = \mu_{max} - 0.61$$

$$\mu_{max} = 0.37\ h^{-1}$$

## 5.8 APPENDIX 8

Specific Substrate uptake rate ( $Q_s$ )

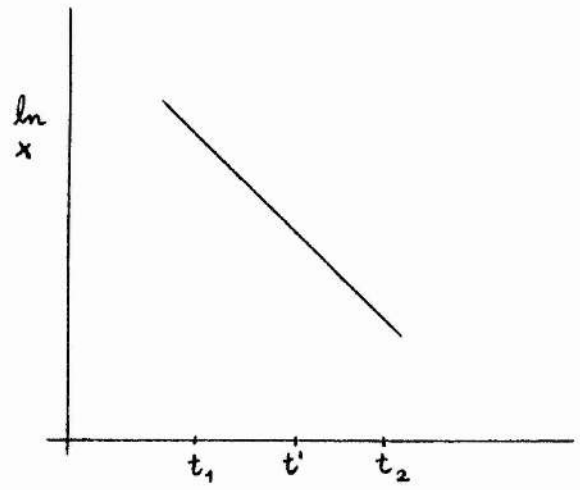
The substrate consumption rate in the batchwise culture has been calculated for the exponential phase of growth according to:

$$\Delta s/\Delta t = Q_s . X^{aver}$$

where $\Delta s/\Delta t$ (g/l/h) is the slope of the straight line produced when substrate concentration is plotted against time.

$Q_s$ is the specific substrate uptake rate ($h^{-1}$)

$X^{aver}$ (g/l) is the average biomass concentration at the time (t') defined by $t_1 + \Delta t /2$, according to the figure




where $t_1$ and $t_2$ are interval time of the exponential phase and $t = t_2 - t_1$.

## 5.9 APPENDIX 9

Specific rate of ethanol formation ($Q_p$)

The ethanol formation rate in the batchwise culture has been calculated for the exponential phase of growth according to:

$$\Delta p/\Delta t = Q_p \cdot X^{aver}$$

Where $\Delta p/\Delta t$ (g/l/h) is the slope of the straight line produced when product concentrations are plotted against time.

$Q_p$ (g ethanol/g cells/h) is the specific rate of ethanol formation.

$X^{aver}$ (g/l) is the average biomass concentration at the time (t') defined by $t_1+t_2/2$ according to the figure in appendix 8

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